PATHOGENESIS, DIAGNOSIS AND TREATMENT OF LYME AND OTHER TICK-BORNE DISEASES

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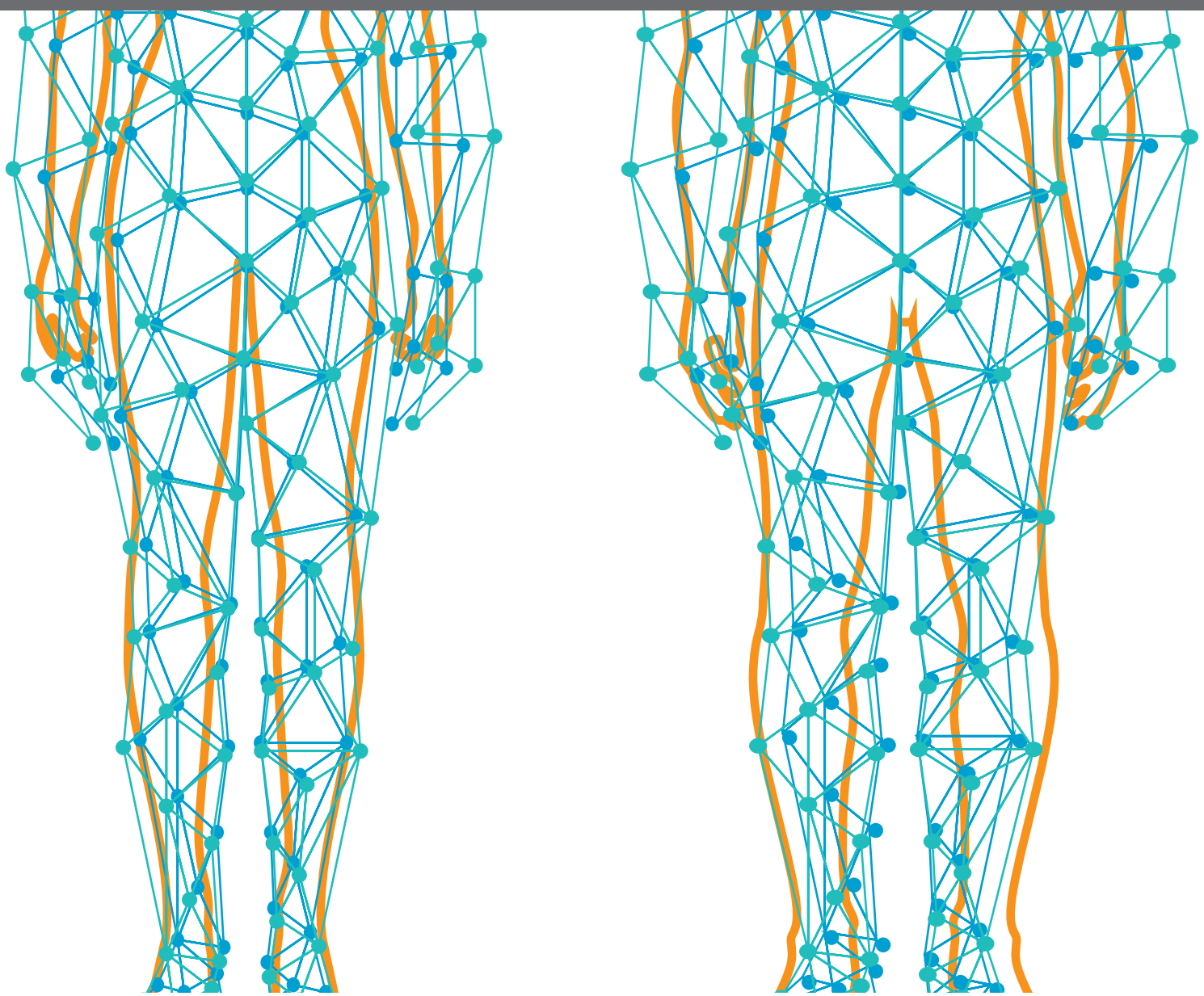

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PATHOGENESIS, DIAGNOSIS AND TREATMENT OF LYME AND OTHER TICK-BORNE DISEASES

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Evaluation of Patient IgM and IgG Reactivity Against Multiple Antigens for Improvement of Serodiagnostic Testing for Early Lyme Disease

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Serologic testing is the standard for laboratory diagnosis and confirmation of Lyme disease. Serodiagnostic assays to detect antibodies against *Borrelia burgdorferi*, the agent of Lyme borreliosis, are used for detection of infection. However, serologic testing within the first month of infection is less sensitive as patients' antibody responses continue to develop. Previously, we screened several *B. burgdorferi in vivo* expressed antigens for candidates that elicit early antibody responses in patients with Stage 1 and 2 Lyme disease. We evaluated patient IgM seroreactivity against 6 antigens and found an increase in sensitivity without compromising specificity when compared to current IgM second-tier immunoblot scoring. In this study, we continued the evaluation using a multi-antigen panel to measure IgM plus IgG seroreactivity in these early Lyme disease patients' serum samples. Using two statistical methods for calculating positivity cutoff values, sensitivity was 70 and 84–87%, for early acute and early convalescent Lyme disease patients, respectively. Specificity was 98–100% for healthy non-endemic control patients, and 96–100% for healthy endemic controls depending on the statistical analysis. We conclude that improved serologic testing for early Lyme disease may be achieved by the addition of multiple borrelial antigens that elicit IgM and IgG antibodies early in infection.

Keywords: *Borrelia burgdorferi*, Lyme disease, serodiagnostics, multiantigen testing, *in vivo*-expressed antigens

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INTRODUCTION

Accurate diagnostic testing for Lyme disease in the early stages of infection is important to deliver proper antibiotic treatment to patients thereby avoiding serious complications that can arise if untreated. Infection by *Borrelia burgdorferi*, the tick-borne bacterial agent of Lyme disease, progresses over three stages: Stage 1; early localized, characterized by a rash (termed erythema migrans) at the tick bite site; Stage 2; early disseminated, characterized by colonization of tissues and organs producing symptoms including myalgia, arthralgia, with acute cardiac or neurologic involvement; and Stage 3; late disseminated, characterized by arthritis and neurological symptoms (1). Antibiotic therapy is effective when administered at all stages, but early treatment following onset of illness represents the best course for successful cure. Based on subjective symptoms similar to several illnesses (e.g., fever, fatigue), clinical diagnoses can be challenging. Patients that

exhibit an erythema migrans (EM) rash at the tick bite site and live in regions of endemicity (i.e., habitats where *Ixodes scapularis*, the tick vector for *B. burgdorferi*, resides) are considerations for a correct diagnosis and prompt treatment. *B. burgdorferi* infection does not produce a bacteremia with abundant organisms in the bloodstream, therefore diagnostic testing by culture, microscopic examination, or PCR is not presently feasible. Current laboratory diagnostic tests rely on the detection of anti-*B. burgdorferi* antibodies to indicate patient exposure to this tick-transmitted spirochete, therefore a confirmation of Lyme disease depends on accurate serologic assays that consider the pretest likelihood and thus the predictive value of laboratory tests.

The current serologic testing recommendation from the Centers for Disease Control and Prevention is a two-step approach with the first being an ELISA of a whole cell sonicate or a peptide of *B. burgdorferi*. When this step yields a positive or indeterminate result, the second step consists of the more specific immunoblot (https://www.cdc.gov/lyme/diagnosistesting/labtest/twostep/index.html). Modifications of the first- and second-tier tests that use combinations of whole cell or recombinant borrelial antigens have been cleared by the U.S. Food and Drug Administration and are commercially available for clinical testing (2). However, sensitive serologic testing is limited during the first days, usually <30, after the patient has been subjected to an infected tick bite, as the full antibody repertoire has not developed (3, 4).

Our attempt to improve the sensitivity of serologic assays in patients with early Lyme disease is based on two hypotheses. First, that IgM and IgG antibodies are produced against a set of antigens that are presented by the host's adaptive immune system in the first days following infection. Second, that there are borrelial antigens expressed *in vivo* within the tick or human hosts that are not present in culture-grown whole cell protein lysate, thereby representing targets for early antibodies. Previously, we screened several antigens that were known to be expressed *in vivo* in ticks and mammalian hosts against a panel of Lyme disease patient serum samples and controls (5). The antigens BBA65, BBA70, and BBA73 were selected for IgM serum immunoreactivity evaluation in early Lyme disease patients together with the three antigens currently used in IgM second-tier immunoblotting, OspC, BmpA, and FlaB. We found that a six antigen approach, whereby reactivity against at least 2 of 6 antigens constituted a positive serology, could increase sensitivity without compromising specificity (6). Also in our initial screening of antigens, BBA69 and BBA73 demonstrated IgG reactivity in a set of early Lyme disease patient samples (5).

In this study, we evaluated IgG seroreactivity against the gene products BBA69 and BBA73 together with antigens OspC, DbpA, FlaB, and VlsE in Stage 1 and Stage 2 early Lyme disease patient serum samples, and combined IgM and IgG responses in a multi-antigen approach for sensitivity and specificity determination. We applied two statistical approaches, one of which evaluates all antigens simultaneously and may select different antigen combinations depending on disease category to maximize performance.

MATERIALS AND METHODS

Recombinant Protein Expression and Purification

Truncated (i.e., lacking signal sequence and lipidation motif) genes encoding BBA69, BBA73, OspC, and DbpA were amplified by PCR from *B. burgdorferi* strain B31 genomic DNA using primers described previously (5, 6). Recombinant proteins were generated and purified in soluble form in *Escherichia coli* with the pETite N-His vector following the T7 Expresso system instructions (Lucigen, Middleton, WI). Cloned genes in expression plasmids were transformed into *E. coli* 10G (Lucigen) and selected for growth on Luria-Bertani (LB) medium plates supplemented with 50 ug/ml kanamycin.

Plasmid DNA from transformant colonies was purified by miniprep (Qiagen, Valencia, CA) and was sequenced for insert confirmation. Recombinant plasmids with the correct gene inserts were transformed into *E. coli* BL21(DE3) (Lucigen). Following transformant screening for the appropriate clones, colonies were grown in LB-kanamycin (50 ug/ml) broth, and recombinant protein expression was induced by the addition of isopropyl-D-thiogalactopyranoside (IPTG; 1 mM). Cells were harvested at late-log-phase growth, and recombinant protein was purified under non-denaturing conditions using a nickel-nitrilotriacetic acid (Ni-NTA) Fast Start His tag affinity purification kit (Qiagen). FlaB does not contain a signal sequence, therefore the entire coding sequence was amplified, cloned, and expressed as described (6). The FlaB protein was purified following manufacturer's instructions for preparation of insoluble protein. Proteins were dialyzed into PBS (pH 7.4) and quantified by bicinchoninic acid (BCA) assay (Thermo-Fisher Scientific, Rockford, IL) before use. Purity of recombinant proteins was assessed by SDS-PAGE staining as demonstrated previously (5). Cloning, expression and purification of recombinant VlsE was performed as previously described with the final product dialyzed in PBS (7).

ELISA

Recombinant antigens were diluted with carbonate buffer (90 mM $NaHCO_3$, 60 mM Na_2CO_3; pH 9.6) and bound to 96-well Immulon 2HB format plates overnight at 4°C (Thermo Scientific, Rockford, IL) at a final concentration of 200 ng/well. The plate wells were subjected to five washes with Tris-buffered saline–Tween 20 [TBS-T; 20 mM Tris, 140 mM NaCl, 2.7 mM KCl, 0.05% Tween 20 (pH 7.4)] using a BioTek 405 Select plate washer (BioTek, Winooski, VT), followed by addition of blocking buffer (TBS-T with 3% fetal bovine serum) for 45 min at room temperature. Serum samples were diluted 1:100 in blocking buffer, then added to the wells coated with the antigens, and the plates were incubated for 60 min with moderate agitation at room temperature followed by five washes with TBS-T. Alkaline phosphatase-conjugated goat anti-human IgG (H + L, KPL, Gaithersburg, MD) was added at 1:5,000 in TBS, and plates were incubated for 45 min. with agitation at room temperature followed by the wash step. For development, 100 μL of para-nitrophenyl phosphate (PNPP) substrate (Thermo-Fisher Scientific) was added to each well, followed by incubation

with agitation at room temperature for 20 min. The reaction was stopped by adding 50 μL of 2 N NaOH to wells. Plates were read at an optical density at 405 nm (OD405) using an ELx808IU Ultra microplate reader (BioTek). Each serum sample was assayed in duplicate. Optimal antigen, serum and conjugate dilutions were determined prior to running the samples as described previously (5). A moderately-reactive serum sample to BBA73 was used as a positive control for each plate, and a low-reactive serum sample to the same antigen was used as a negative control. Optical density data was recorded and used for statistical analysis. Serum sample IgM optical density data was previously performed and recorded as described (6).

Serum Samples

The Lyme Serum Repository (LSR) was the source of human serum panels used in this study, and samples were collected by the Division of Vector Borne Diseases, Bacterial Diseases Branch, Centers for Disease Control and Prevention. A detailed description of the LSR, which is composed of serum obtained from well-characterized Lyme disease patients, control serum from healthy individuals, and serum from patients with other diseases, has been published (8). Lyme disease patient samples were subdivided into groups as follows: early Lyme disease with EM, which consisted of paired patient serum samples taken at the acute and convalescent phases of disease (stage 1; $n = 78$); early Lyme neuroborreliosis (stage 2; $n = 9$); and early Lyme carditis (stage 2; $n = 7$). Patients with early Lyme disease with EM could be scored as two-tiered negative, but for acceptance into the serum panel, they were required to have well-documented clinical and laboratory (PCR and/or culture) evidence of infection.

This study was carried out in accordance with the recommendations of the Institutional Review Board (IRB) & Research Determinations, Human Studies Team, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Centers for Disease Control and Prevention. The protocol was approved by the NCEZID IRB board and determined that it does not include human subjects, as defined under 45 CFR 46.102(f). IRB review was not required. Informed consent and Institutional Review Board approval was granted for the testing of these samples.

Statistical Analyses and Cutoff Calculations

To normalize for anticipated daily variation of the assay measurements, duplicate positive control wells employing a reactive serum control against rBBA73 were included on each plate. Optical density (OD) values were normalized by dividing all OD values on the plate by the positive plate controls' average OD. Exploratory analysis showed relatively little variance attributable to user or date replications, therefore sample replicates were averaged over these prior to further analysis. Natural logarithms (ln) of the normalized values were computed for use as the primary measure in analyses (data shown in **Figure S1**).

Upon closer examination of the original data, six of the healthy endemic samples had abnormally high OD values. A follow-up principal components analysis indicated that these samples were indeed outside of the normal range for a typical healthy endemic and so were excluded as controls.

Two methods were used to calculate cutoffs to declare samples positive for *B. burgdorferi* infection. The first method of cutoff determination used a receiver operating characteristic (ROC) curve for each antigen being tested. We selected the cutoff that maximized sensitivity while fixing specificity at 99%. A positive result for the serum sample was declared whenever 2 or more of the 12 antigen measurements (6 antigens for IgM and 6 antigens for IgG) were above their respective cutoffs. For sensitivity of each disease group, we compared the samples to the healthy non-endemic samples. Specificities were calculated by applying the computed antigen cutoffs for the early EM acute group to the healthy and non-Lyme disease samples.

The second method of cutoff determination was to compute a "score" for each sample in a logistic regression model that combined the normalized ln(OD) values for all 12 antigens. For each disease category compared to healthy non-endemic patient samples, we computed the scores by finding the weights for the linear combination (i.e., weighted sum) of normalized ln(OD) values for all antigens that maximized the area under the ROC curve (AUC) (9). Generalized cross-validation (GCV) was used for each of these fits to provide a more robust estimate of the AUC for each linear combination. The linear combinations were computed for each possible subset of antigens (4095 possible sets of the 12 antigens) and ranked by their GCV-AUC values. The top-ranked linear combination was the one with the highest GCV-AUC, and the associated scores from these were then used in ROC analyses to determine the cutoff that maximized sensitivity while fixing specificity at 99%. The cutoff value obtained for all early Lyme disease group samples (i.e., EM acute, EM convalescent, neuroborreliosis, and carditis) was used to determine specificity for the non-Lyme disease and healthy endemic categories combined. We computed 95% confidence intervals (CIs) for sensitivity and specificity when using both methods. The coefficients determine how each antigen OD value is included in overall score of the linear combination. A negative coefficient lowers the score, while a positive coefficient increases it. Because the antigen OD values are scaled within-antigen, the coefficients can also be compared for relative effect, so that, for example, a coefficient of 0.48 is 4 times as impactful as one of 0.12, for the same OD value. When no coefficient listed in the table, its coefficient is 0, meaning that particular antigen does not contribute to the ROC-AUC linear combination for that category.

RESULTS

ELISA IgM and IgG Combined Evaluation of Early Lyme Disease Patient Serum Samples Against 12 Antigens

Setting Cutoff Values Using Receiver Operator Characteristic (ROC) Curve Analysis

We analyzed the data by setting cutoff values for IgM plus IgG positivity by ROC curve analysis of the healthy non-endemic control serum patient samples vs. each disease group samples.

TABLE 1 | IgM plus IgG sensitivity and specificity of early Lyme disease patient samples.

Patient category	*N*	No. positive (% Sensitivity) [95% CI]
Lyme disease		**≥ 2 antigens positive**
Early EM acute	40	28 (70) [55–82]
Early EM convalescent	38	32 (84) [70–93]
Carditis	7	6 (86) [49–99]
Neuroborreliosis	9	8 (89) [57–99]
Non-Lyme disease		**No. positive (% Specificity) [95% CI]**
		<2 antigens positive
Fibromyalgia	31	1 (97) [84–100]
Mononucleosis	30	4 (87) [70–95]
Multiple sclerosis	21	3 (86) [65–95]
Periodontitis	20	1 (95) [76–100]
Rheumatoid arthritis	21	1 (95) [77–100]
Syphilis	20	8 (60) [39–78]
Healthy endemic	94	4 (96) [90–98]
Healthy non-endemic	102	2 (98) [93–99]

N, number of samples.
ROC cutoffs based on 99% specificity for healthy non-endemic samples.

Specificity was set at 99% when determining the ROC cutoff. **Table 1** shows the sensitivities for each Lyme patient category with reactivity to ≥2 of the 12 antigens scored as positive.

Sensitivity for the early EM acute patients was 70% (28/40), with 84% (32/38) of the paired samples representing early EM convalescent testing positive. Sensitivity was 86% (6/7) for the carditis patients, and 89% (8/9) for the neuroborreliosis patients. Although specificity was set at 99% for each individual antigen's ROC cutoff, the specificity for the combined antigen method (<2 positive antigens) for the healthy non-endemic patients was calculated at 98% (100/102) due to the discrete nature of the data. Specificity for the healthy endemic patients, however, was lower at 96% (90/94). The non-Lyme disease patient samples demonstrated a range of specificities from 60 to 97%, with the lowest being syphilis patients (**Table 1**).

Setting Cutoff Values by Linear Combination of Antigen Normalized ln(OD) Values Maximizing the ROC AUC

The second method of deriving cutoffs used the scores calculated from the linear combination of normalized ln(OD) values that maximized the AUC and gave the coefficients (weights) corresponding to the highest GCV-AUC listed for each Lyme disease category (**Table 2**). Each disease category was compared to healthy non-endemic patient samples. The estimate of the GCV-AUC is shown, as is the positivity cutoff-value determined using the ROC curve derived from the score value for each sample, using the coefficients shown. The estimated sensitivities and 95% CIs using the positivity cutoff given are also shown in **Table 2**.

Early EM acute Lyme disease was evaluated and demonstrated that the full subset of antigens (except BmpA) resulted in a GCV-AUC of 0.97, and the corresponding coefficients (weights) for

TABLE 2 | Linear combination giving the highest cross-validated AUC.

Patient category	*N*	No. positive (% Sensitivity) [95% CI]	Cutoff value	GCV-AUC	Coefficients										
					IgM						IgG				
					BmpA	FlaB	OspC	BBA65	BBA70	BBA73	DbpA	FlaB	OspC	VlsE	BBA69	BBA73
Early Lyme EM Acute	40	28 (70) [55–82]	0.94	0.97		0.23	−0.18	−0.29	0.09	−0.16	0.11	0.49	0.42	0.61	−0.01	0.02
Early Lyme EM Convalescent	38	33 (87) [73–94]	1.03	0.96	−0.11	0.06	0.14	−0.34		0.03		0.43		0.79	−0.15	−0.03
Carditis	7	7 (100) [65–100]	0.65	1.00	0.10	−0.08	0.54		−0.26		0.56	0.25		0.49		
Neuroborreliosis	9	9 (100) [70–100]	0.08	1.00		0.64	0.61	−0.25	−0.31	0.22					0.06	−0.02
Non-Lyme disease		**No. positive (% Specificity a) [95% CI]**														
Healthy non-endemic	102	1 (99) [65–100]														

[a]*Specificity based on early Lyme EM acute linear combination and cutoff.*

the antigens are shown in **Table 2**. The positivity cutoff value for the scores computed using these coefficients was 0.94, which resulted in an estimated sensitivity of 70% (28/40) (**Table 2**). Sensitivity was similarly calculated for early Lyme disease convalescent samples and resulted at 87% (33/38). Sensitivity for neuroborreliosis samples was 100% (7/7), and was 100% (9/9) for carditis samples (**Table 2**).

Specificities were calculated by comparing all early Lyme disease samples to all non-Lyme disease and healthy endemic samples combined (**Table 3**). Specificities for the non-Lyme disease samples ranged from 95 to 100% with only 2 false positives; one each in the syphilis and multiple sclerosis groups. Specificity for the healthy endemic patient samples was 100% (**Table 3**).

Breakdown of Number of Positive Antigens per Serum Sample Tested

An interesting observation during the ROC analysis of the data was the number of individual serum samples that were positive for at least 3 antigens. As noted in **Table 4**, several early Lyme disease patients scored positive for 3–7 antigens with some patients showing reactivity to 8–10 antigens. This result indicates how individual patients elicit antibodies early following infection against an array of borrelial antigens. The observation shown in **Table 4** indicates the potential to generate improved serological testing utilizing multiple antigens in a combined IgM plus IgG serological assay.

DISCUSSION

Several studies have reported on modified serodiagnostic assays for early Lyme disease evolving from standard two-tier testing suggesting a number of approaches to improve sensitivity while maintaining specificity (2, 10–12).

In this study we assessed a multi-antigen strategy to detect IgM and IgG antibody responses in patients with early onset of infection. We hypothesized that antigens synthesized by *B. burgdorferi in vivo* and processed early by the immune system would provide additional targets for detection of the first wave of antibody production. In this study, we combined the 6 antigens described in our previous work for improving IgM serology (BmpA, FlaB, OspC, BBA65, BBA70, and BBA73) with 2 antigens we identified as IgG reactive, BBA69, and BBA73 (5, 6). We also included VlsE, DbpA, FlaB, and OspC for the IgG analysis as these antigens have been documented as seroreactive in patients with early Lyme disease (7, 13, 14). FlaB, OspC, and BBA73 were tested for both IgM and IgG in this study.

We used two statistical approaches to set ELISA cutoff values and calculated sensitivity and specificity based on combined seroreactivity by IgM plus IgG (6 antigens each). In our previous study, we found that the ROC and ROC-AUC provided the most robust computational analyses (6). Consistent with that study, we set cutoffs using the healthy non-endemic patient serum as controls.

With both statistical methods, we found sensitivities of 70 and 84–87% for early acute and early convalescent Lyme disease patients, respectively, using the combined 12 antigen IgM and

TABLE 3 | Specificities based on all early Lyme against all non-Lyme diseases and healthy endemics.

Patient category	N	No. positive (% Sensitivity) [95% CI]	Cutoff value	GCV	Coefficients											
					IgM						IgG					
					Bmp A	FlaB	OspC	BBA65	BBA70	BBA73	DbpA	FlaB	OspC	VlsE	BBA69	BBA73
All early Lyme	94	67 (71) [61.4–79.4]	1.21	0.93	−0.14	0.29	0.37		−0.44		−0.04	0.13	0.10	0.72		−0.14
Non-Lyme disease	**N**	**No. positive (% Specificity) [95% CI]**														
Fibromyalgia	31	0 (100) [89–100]														
Mononucleosis	30	0 (100) [89–100]														
Multiple sclerosis	21	1 (95) [77–100]														
Periodontitis	20	0 (100) [84–100]														
Rheumatoid arthritis	21	0 (100) [85–100]														
Syphilis	20	1 (95) [76–100]														
Healthy endemic	94	0 (100) [96–100]														

TABLE 4 | Number of antigens scored positive per individual serum sample by ROC analysis.

No. positive antigens[a]	0	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Sum**
LYME DISEASE												
Early Lyme EM Acute	7	5	**7**	**4**	**4**	**4**	**4**	**1**	**1**	**1**	**2**	40
Early Lyme EM Convalescent	4	1	**6**	**2**	**3**	**7**	**5**	**6**	**2**	**1**	**1**	38
Carditis	0	0	**2**	**0**	**1**	**2**	**0**	**0**	**1**	**0**	**1**	7
Neuroborreliosis	0	1	**0**	**1**	**1**	**1**	**1**	**1**	**2**	**1**	**0**	9
Sum	11	7	**15**	**7**	**9**	**14**	**10**	**8**	**6**	**3**	**4**	
NON-LYME DISEASE												
Fibromyalgia	21	9	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	31
Mononucleosis	17	9	**3**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	30
Multiple sclerosis	16	2	**3**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	21
Periodontitis	16	3	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	20
Rheumatoid arthritis	16	4	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	21
Syphilis	5	7	6	2	0	0	0	0	0	0	0	20
Healthy endemic	84	6	3	1	0	0	0	0	0	0	0	94
Healthy non-endemic	94	6	2	0	0	0	0	0	0	0	0	102

[a]*No Lyme disease patient samples scored positive for 11–12 antigens.*
The bold numbers signify no. of antigens required for a positive score.

IgG seroreactivities. These results are increased over the standard 2-tiered testing for these samples at 40 and 61% (8). Our sensitivity results also compare favorably and in some cases are higher than published reports for early Lyme disease detection (10), however it is difficult to compare as these studies used different serum samples than were used here. When the same serum samples were used, our sensitivities were increased over results reported for standard two-tiered tests (STTTs) for both early acute samples (70 vs. 40–48%) and for early convalescent samples (84–87 vs. 61–68%) (2). We also found increased sensitivities over results reported for 2 modified two-tiered tests (MTTTs), i.e., 2-EIA approaches, for early acute samples (70 vs. 48–50%) and early convalescent samples (87 vs. 74–79%) (2). A second study by Pegalajar-Jurado et al., evaluated 3 additional 2-EIA MTTTs against the serum samples used in our study with sensitivities for early acute samples from 50 to 58%, and from 76 to 79% for early convalescent samples, both lower than our results (12).

When calculated by ROC, however, our specificity was lower for healthy endemic serum samples at 96%, with increases in false positives for non-Lyme diseases compared to the STTTs and MTTTs. This result may be reasonably tolerated as clinical diagnoses should differentiate Lyme disease from syphilis and periodontal disease for example. We note that only 1 false positive each for rheumatoid arthritis and fibromyalgia samples were scored, both diseases that could be misdiagnosed as Lyme disease.

Specificities estimated by the more sophisticated ROC-AUC statistical approach resulted in exceptional values of 100% for healthy endemic patients and all non-Lyme patients (except for syphilis and multiple sclerosis which only had one false positive each). Utilization of the ROC-AUC methodology would be useful with an unrestricted, well-studied number of antigens and a sufficiently large set of serum samples where such an algorithm has the potential to maximize the value of the data for sensitivity and specificity by finding the best combination of antigens for each disease category. We showed resultant specificities for the non-Lyme categories based on the cutoff score for all early Lyme categories as an example of the usefulness of this methodology (**Table 3**).

Combined testing for IgM with IgG resulted in much greater sensitivity than we previously reported for IgM alone. For early acute Lyme samples, sensitivity increased to 70% from 28 to 30% testing with IgM only. For early convalescent Lyme samples, sensitivity increased to 84–87% from 50 to 68% with IgM only (6).

An interesting finding was the number of individual patients in the early stages of infection that reacted positively with 3 or more antigens, and in some cases up to 6–10 antigens. Obviously, although specificity with this number of positives would be nearly 100%, sensitivity would be below an acceptable threshold.

This result suggests that (i) individual patients are unique in their elicitation of antibodies against a spectrum of borrelial antigens, and (ii) infectious Borrelia populations express or harbor a differential array of antigenic proteins which may be amenable to host processing. This finding suggests potential for improved serological testing utilizing multiple antigens in a combined IgM plus IgG serological assay.

In conclusion, several investigations using the multi-antigen approach to improve serologic testing for Lyme disease have been reported underscoring the rationalization for adding antigens for new test algorithms (14–18). A commercial assay would likely employ multiplex testing technology to enhance sensitivity over ELISA formats and provide a platform to screen dozens of antigens (11, 19, 20). These studies and ours represent pilot versions of algorithms for new tests and warrant validation with higher numbers of prospectively and retrospectively collected patient samples.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

KB and RG conceived and carried out the experiments. KB, RG, KH, and BB analyzed the data. KH and BB performed the statistical analyses. RG concepted and supervised the study. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2019.00370/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The General Symptom Questionnaire-30 (GSQ-30): A Brief Measure of Multi-System Symptom Burden in Lyme Disease

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Introduction: The multi-system symptoms accompanying acute and post-treatment Lyme disease syndrome pose a challenge for time-limited assessment. The General Symptom Questionnaire (GSQ-30) was developed to fill the need for a brief patient-reported measure of multi-system symptom burden. In this study we assess the psychometric properties and sensitivity to change of the GSQ-30.

Materials and Methods: 342 adult participants comprised 4 diagnostic groups: Lyme disease (post-treatment Lyme disease syndrome, $n = 124$; erythema migrans, $n = 94$); depression, $n = 36$; traumatic brain injury, $n = 51$; healthy, $n = 37$. Participants were recruited from clinical research facilities in Massachusetts, Maryland, and New York. Validation measures for the GSQ-30 included the Patient Health Questionnaire-4 for depression and anxiety, visual analog scales for fatigue and pain, the Sheehan Disability Scale for functional impairment, and one global health question. To assess sensitivity to change, 53 patients with erythema migrans completed the GSQ-30 before treatment and 6 months after 3 weeks of treatment with doxycycline.

Results: The GSQ-30 demonstrated excellent internal consistency (Cronbach $\alpha = 0.95$). The factor structure reflects four core domains: pain/fatigue, neuropsychiatric, neurologic, and viral-like symptoms. Symptom burden was significantly associated with depression ($r_S = 0.60$), anxiety ($r_S = 0.55$), pain ($r_S = 0.75$), fatigue ($r_S = 0.77$), functional impairment ($r_S = 0.79$), and general health ($r_S = -0.58$). The GSQ-30 detected significant change in symptom burden before and after antibiotic therapy; this change correlated with change in functional impairment. The GSQ-30 total score significantly differed for erythema migrans vs. three other groups (post-treatment Lyme disease syndrome, depression, healthy controls). The GSQ-30 total scores for traumatic brain injury and depression were not significantly different from post-treatment Lyme disease syndrome.

Conclusions and Relevance: The GSQ-30 is a valid and reliable instrument to assess symptom burden among patients with acute and post-treatment Lyme disease syndrome and is sensitive in the detection of change after treatment among patients with erythema migrans. The GSQ-30 should prove useful in clinical and research settings to assess multi-system symptom burden and to monitor change over time. The GSQ-30 may also prove useful in future precision medicine studies as a clinical measure to correlate with disease-relevant biomarkers.

Keywords: Lyme disease, GSQ-30, PTLDS, multi-system illness, symptom burden

INTRODUCTION

Lyme disease is a serious and debilitating global illness, with estimated rates exceeding 400,000 new cases annually in the United States alone (1). While most patients recover fully after early detection and treatment, ∼10% have symptoms that last 6 months or longer associated with functional impairment ("Post-treatment Lyme Disease Syndrome" or PTLDS) (2–4). Patients with persistent symptoms pose a challenge to clinicians as the symptoms vary across multiple medical domains, including the rheumatologic, neurologic, infectious, cardiac, psychiatric, and neurocognitive. This diversity of symptoms can make it difficult to assess treatment progress. Given the clinician's limited time with each patient, a brief self-report screening instrument covering multiple symptom domains would allow a rapid quantification of symptom burden, facilitate monitoring of change over time, and highlight other disease-relevant symptoms that require attention.

There are many somatic symptom scales that include symptoms commonly reported in the primary care setting (5–7). To our knowledge, there is only one self-report instrument specifically developed to address symptoms common to patients with Lyme disease. This instrument—the Horowitz Multiple Systemic Infectious Disease Syndrome Questionnaire—is a measure designed for the primary purpose of diagnosis of Lyme disease and other tick-borne disorders (8).

We designed the General Symptom Questionnaire (GSQ-30) to fill the need for a brief self-report instrument that assesses symptom burden and response to treatment among patients with multi-system disease. This instrument would be valuable in clinical trials and provide a quantitative clinical index for assessing the clinical relevance of biomarkers. While the GSQ-30 may be useful for monitoring a variety of multi-system medical conditions, it was designed specifically for patients with Lyme disease. In conducting this validation study, we hypothesized that the GSQ-30 would have good psychometric properties, be sensitive to detecting change after antibiotic treatment, and demonstrate clinically relevant profile differences between early and post-treatment Lyme disease symptoms and between Lyme disease and health. As a secondary goal, we examined whether the profile of PTLDS would differ from two similarly disabling conditions with multi-system symptoms- depression and traumatic brain injury (TBI).

MATERIALS AND METHODS

Participants

Three hundred and forty-two participants, recruited across multiple sites, included 94 with early Lyme Disease who had a health-care provider diagnosed erythema migrans (EM) rash ($n = 12$ from the Lyme Center at Columbia University; $n = 82$ from the Lyme Center at Johns Hopkins University), 124 with IDSA case-defined PTLDS ($n = 30$ from Columbia; $n = 94$ from Johns Hopkins), 36 with depression from the New York State Psychiatric Institute (NYSPI), 51 with TBI from the outpatient brain injury clinic at Harvard's Spaulding Rehabilitation Hospital, and 37 healthy control participants ($n = 14$ from Columbia; $n = 23$ from Johns Hopkins).

The patients with EM had a rash with or without disseminated symptoms at study entry. The PTLDS patients met the IDSA case-definition which requires persistent symptoms that emerged during the first 6 months after antibiotic therapy for well-documented Lyme disease (4). The depressed participants had to score 14 or higher on the BDI-II indicating at least mild depression ($M = 30.11$, $SD = 9.29$). The TBI participants had to have a Glasgow Coma Scale score that fell in the mild (14–15) to moderate (9–13) range at least 18 months post-injury. Neither the depressed patients nor the TBI patients had a known history of Lyme disease. The healthy control participants were seronegative for *Borrelia burgdorferi* antibodies and free of symptoms associated with Lyme disease, medically healthy (Columbia site) or medically stable (Johns Hopkins site), had no history of major medical illness or severe viral-like symptoms in the last 6 months, and had no prior diagnosis or treatment for a tick-borne illness.

Measures

The GSQ-30 is a 30 item questionnaire which assesses symptom burden over a 2 week time period (see **Figure 1**). Modeled after measures of somatic symptom burden in primary care, the PHQ-15 (5) and the SSS-8 (6), the GSQ-30 asks: "how much have you been bothered by any of the following?" with five options: "not at all," "a little bit," "somewhat," "quite a bit," and "very much" (scored 0–4); total score ranges from 0 to 120. The 2 week timeframe was selected to be shorter than the 1 month interval used for the PHQ-15 to minimize recall bias, and longer than the 1 week interval used for the SSS-8 to account for the waxing and waning nature of Lyme disease symptoms. The items

NAME (or ID) ___ ___ ___ ___ ___ ___ Date: _____/_____/_____

SYMPTOMS. During the past 2 weeks, how much have you been **bothered** by any of the following?

Rate "bother" for the past 2 weeks	Not at all	A little bit	Somewhat	Quite a bit	Very much
1. Shortness of breath	0	1	2	3	4
2. Feeling feverish	0	1	2	3	4
3. Sweats and/or chills	0	1	2	3	4
4. Nausea and/or vomiting	0	1	2	3	4
5. Back pain	0	1	2	3	4
6. Headaches	0	1	2	3	4
7. Stiff or painful neck	0	1	2	3	4
8. Muscle aches or pains	0	1	2	3	4
9. Joint pain or swelling	0	1	2	3	4
10. Muscle weakness	0	1	2	3	4
11. Feeling fatigued or having low energy	0	1	2	3	4
12. Feeling worse after normal physical exertion	0	1	2	3	4
13. Trouble falling or staying asleep	0	1	2	3	4
14. Needing more sleep than usual	0	1	2	3	4
15. Not feeling rested on awakening	0	1	2	3	4
16. Numbness or tingling	0	1	2	3	4
17. Shooting, stabbing or burning pains	0	1	2	3	4
18. Skin or muscle twitching	0	1	2	3	4
19. Discomfort with normal light or sound	0	1	2	3	4
20. Balance problems or sense of room-spinning	0	1	2	3	4
21. Change in visual clarity or trouble focusing	0	1	2	3	4
22. Bladder discomfort or change in urination	0	1	2	3	4
23. Light-headed or uncomfortable on standing	0	1	2	3	4
24. Hot or cold sensations in extremities	0	1	2	3	4
25. Irregular or rapid heart beats	0	1	2	3	4
26. Feeling irritable, sad, or decreased pleasure	0	1	2	3	4
27. Feeling panicky, anxious or worried	0	1	2	3	4
28. Trouble finding words or retrieving names	0	1	2	3	4
29. Trouble with memory	0	1	2	3	4
30. Slower speed of thinking	0	1	2	3	4

Over the last 2 weeks, have any of the above impaired your work, social, or family functioning? Yes No

If Yes, please indicate the number (#) of each of the most impairing symptoms below**, starting with the most impairing (#1)**, then list the next most impairing (#2) and continue listing in descending severity other impairing symptoms.

1. _____ 2. _____ 3_____ 4_____ 5_____ 6_____ 7_____

FIGURE 1 | The general symptom questionnaire (GSQ-30).

selected for the GSQ-30 reflect somatic and neuropsychiatric symptoms commonly reported by patients with Lyme disease as noted in the literature (9–11) and from the authors' clinical research experience (BAF, NZ, JNA).An additional question (not included in the scoring) asks whether any of the above 30 items have impaired work, social or family functioning; the rater then lists the most impairing items in rank order of severity (up to seven items), thereby highlighting symptoms of most concern to the individual.

The Patient Health Questionnaire-4 (PHQ-4) (12) is an ultra-brief four item instrument with good psychometric properties developed to assess anxiety (items 1–2) and depression (items 3–4).

The Sheehan Disability Scale (SDS) (13), a valid and reliable measure of disability (14, 15), is a brief self-report measure designed to assess functional impairment across three domains of work/school, social and family life. The measure was adapted to span functioning "over the past 2 weeks." The summed

score for the three domains provides a measure of global functional impairment.

Visual analog scales (VAS) assessed pain and fatigue over the prior 2 weeks with scores ranging from 0 ("No___") to 10 ("Most Severe___"). Visual analog scales have been shown to be valid and reliable in the assessment of pain (16), and are used in studies of fatigue (17, 18).

A single item, rated on a 5-point scale, was used to assess self-reported general health ("excellent," "very good," "good," "fair," or "poor"). This identical item is used in the SF-36 and the CDC HRQOL-4 Module (19, 20). Single item health questions are widely used in population-based research with demonstrated validity and reliability (21).

Procedures

The GSQ-30 was included in ongoing IRB-approved research protocols at NYSPI and Johns Hopkins University during which all participants provided written informed consent. The Spaulding Rehabilitation Hospital/Partners Healthcare IRB authorized the retrospective collection of de-identified clinical data. The NYSPI/Columbia site served as the data coordinating center. All participants completed baseline self-report and demographic questionnaires. A subset of the individuals with EM from the Johns Hopkins site ($n = 53$) completed questionnaires again 6 months after treatment as part of a larger longitudinal cohort study. The pre-treatment and 6 month timepoints were used to assess change.

Statistical Analysis

Internal consistency was assessed using Cronbach's alpha for the total GSQ-30 scale.

Construct validity was examined in three stages. (1) Bivariate correlations were examined between the GSQ-30, PHQ anxiety and depression totals, visual analog scales for pain and fatigue, the SDS total, and the general health item. Due to violations of normality, Spearman rank correlations were conducted with bootstrapped confidence intervals. (2) Sequential multivariable regression was employed, with functional impairment as the outcome variable, GSQ-30 as the predictor, and anxiety and depression scores entered at step 1 as covariates, to determine if symptom severity on the GSQ-30 improved prediction of functional impairment beyond the effects of anxiety/depression. Since regression diagnostics indicated some evidence of heteroscedasticity, a bootstrapped regression model was conducted. (3) Welch one-way tests with Holm correction for multiple comparisons were conducted to examine whether the GSQ-30 total could be used to distinguish between: (a) health status group based on the general health assessment; and (b) PTLDS, EM, depression, TBI, and healthy controls.

Factorial validity was examined with all participants except for healthy controls ($n = 305$) using principal components analysis (PCA) with an oblique "Promax" rotation to identify the number of components and determine the factor structure. PCA was conducted using polychoric correlations due to the ordinal nature of the data (22–24). The Kaiser-Meyer-Olkin measure of sampling adequacy was used to determine adequacy of sample size, and Bartlett's test of sphericity was used to assess suitability of the data for PCA. The number of components was determined by examining the number of eigenvalues >1, scree plot, parallel analysis (25) and significant factor loadings.

Sensitivity to change of the GSQ-30 was assessed in the subsample of patients with EM followed over time ($n = 53$). Treatment response at 6 months was categorized into three

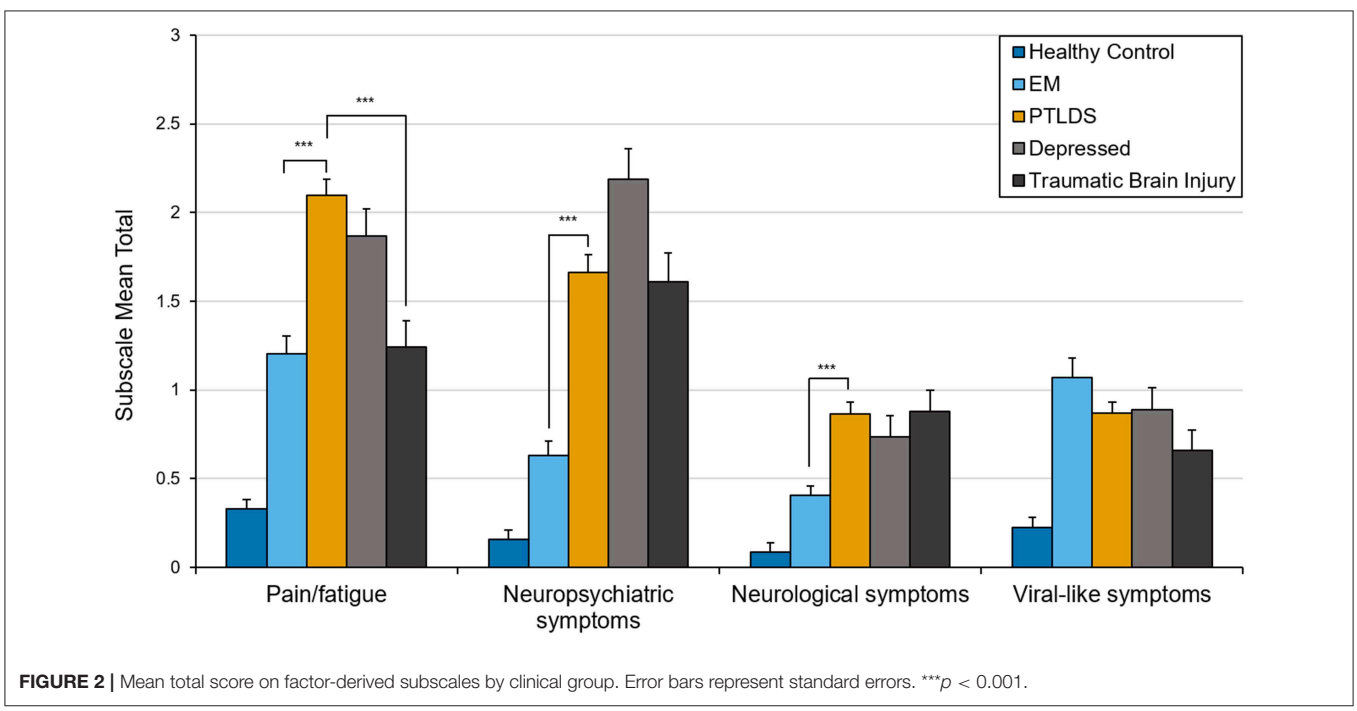

FIGURE 2 | Mean total score on factor-derived subscales by clinical group. Error bars represent standard errors. ***$p < 0.001$.

groups based on the presence of symptoms and/or functional impact, as previously described (2): "PTLDS" (i.e., symptoms with functional impairment), "Symptoms only" (i.e., symptoms without impairment), and "Returned to health."

GSQ-30 scores before treatment were compared to scores 6 months later using a paired samples *t*-test. Percentage change in score from baseline to 6 months was calculated for 50 of the 53 participants from the EM subsample (three were not included due to baseline scores of 0). Percent change was again calculated separately for each outcome group. Given the non-normality of the data, both mean and median percent changes are reported.

In addition, a cross-sectional comparison was conducted among the outcome groups at 6 months using a Welch one-way test. *Post-hoc* tests with Holm correction for multiple comparisons were conducted to compare groups. A mixed ANOVA was also conducted to explore the interaction of time (i.e., pre- and post-treatment visits) and outcome group. Finally, the association between change in GSQ-30 scores and change in functional impairment scores from baseline to the 6 month follow-up was examined using Kendall's tau-b correlation of difference scores.

In an exploratory analysis to determine whether the clinical profile of PTLDS differs from other clinically ill groups, subscale scores representing the mean of items within each of the 4 clusters identified in the PCA were compared and Welch one-way tests with Games-Howell *post-hoc* pair-wise comparisons were conducted. Group means in **Figure 2** include the healthy sample group to aid in interpretation of clinical data. Paired samples *t*-tests were also conducted to examine change over time in the EM subsample using the newly derived subscales.

Missing data was present on key variables at a rate of <5% and imputed with a sequential hot-deck technique (26). All reported

TABLE 1 | Demographic and clinical characteristics of all groups.

	Depressed (*n* = 36)	EM (*n* = 94)	Healthy control (*n* = 37)	PTLDS (*n* = 124)	Traumatic brain injury (*n* = 51)	Total (*n* = 342)
Age, mean (SD), y	36.78 (11.42)	50.67 (14.93)	44.05 (16.18)	44.80 (15.37)	46.63 (16.97)	45.76 (15.66)
Sex (%)						
Female	23 (63.9)	48 (51.1)	25 (67.6)	54 (43.5)	24 (47.1)	174 (50.9)
Male	12 (33.3)	46 (48.9)	12 (32.4)	70 (56.5)	27 (52.9)	167 (48.8)
Other	1 (2.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.3)
Ethnicity (%)						
Black (non-Hispanic)	1 (2.8)	1 (1.1)	3 (8.1)	3 (2.4)	3 (5.9)	11 (3.2)
Hispanic	4 (11.1)	0 (0.0)	6 (16.2)	4 (3.2)	3 (5.9)	17 (5.0)
Other	7 (19.4)	2 (2.1)	7 (18.9)	5 (4.0)	3 (5.9)	24 (7.0)
White (non-Hispanic)	24 (66.7)	91 (96.8)	20 (54.1)	112 (90.3)	38 (74.5)	285 (83.3)
Not specified	0 (0.0)	0 (0.0)	1 (2.7)	0 (0.0)	4 (7.8)	5 (1.5)
Education (%)						
12 years or less	4 (11.1)	7 (7.4)	6 (16.2)	21 (16.9)	7 (13.7)	38 (11.1)
13 to 16 years	20 (55.6)	37 (39.4)	15 (40.5)	57 (46.0)	9 (17.6)	129 (37.7)
16 years or more	12 (33.3)	50 (53.2)	15 (40.5)	45 (36.3)	9 (17.6)	122 (35.7)
Not specified	0 (0.0)	0 (0.0)	1 (2.7)	1 (0.8)	26 (51.0)	53 (15.5)
Employment (%)[a]						
Disabled	–	1 (1.1)	0 (0.0)	4 (3.2)	51 (100.0)	56 (16.4)
F/T or P/T employed	–	67 (71.3)	22 (59.5)	77 (62.1)	0 (0.0)	166 (48.5)
F/T or P/T student	–	2 (2.1)	4 (10.8)	11 (8.9)	0 (0.0)	17 (5.0)
Homemaker	–	5 (5.3)	2 (5.4)	9 (7.3)	0 (0.0)	16 (4.7)
Other	–	2 (2.1)	0 (0.0)	5 (4.0)	0 (0.0)	7 (2.0)
Retired	–	4 (4.3)	3 (8.1)	5 (4.0)	0 (0.0)	12 (3.5)
Unemployed	–	13 (13.8)	5 (13.5)	13 (10.5)	0 (0.0)	31 (9.1)
Not specified	–	0 (0.0)	1 (2.7)	0 (0.0)	0 (0.0)	37 (10.8)
General health, mean (SD)	2.89 (0.89)	3.85 (0.89)	3.95 (0.66)	2.81 (1.02)	3.12 (1.05)	3.27 (1.06)
Pain, mean (SD)	4.19 (2.45)	2.98 (2.81)	0.76 (0.98)	4.58 (2.25)	3.20 (3.03)	3.48 (2.72)
Fatigue, mean (SD)	6.39 (2.41)	3.91 (3.12)	1.35 (1.36)	5.60 (2.45)	4.25 (3.08)	4.56 (3.00)
Anxiety, mean (SD)	3.17 (2.06)	0.83 (1.41)	0.57 (1.14)	1.66 (1.84)	1.84 (2.06)	1.50 (1.87)
Depression, mean (SD)	3.97 (1.76)	0.70 (1.12)	0.22 (0.85)	1.47 (1.53)	1.92 (2.11)	1.45 (1.81)
Functional impairment, mean (SD)	16.69 (8.40)	8.87 (10.09)	1.05 (2.78)	14.19 (8.93)	10.94 (10.34)	11.09 (9.97)

[a] *Employment was categorized differently for the depression group as: employed/not employed (75%, n = 27; 25%, n = 9).*

p-values were 2-sided, with $p < 0.05$ considered statistically significant. Statistical analyses were performed using R version 3.5.1 (27).

RESULTS

Demographics and clinical characteristics of the total sample and individual groups are presented in **Table 1**.

Internal Consistency

Cronbach's alpha for the GSQ-30 total score was excellent, $r = 0.95$. Internal consistency was high for all groups when assessed separately including the healthy controls, EM, PTLDS, TBI, and depression patients; $r = 0.86$, 0.94, 0.93, 0.96, and 0.93, respectively.

Construct Validity

GSQ-30 total scores including all groups were significantly correlated with depression ($r_s = 0.60$, 95% CI[0.53, 0.66]), anxiety ($r_s = 0.55$, 95% CI[0.48, 0.62]), pain ($r_s = 0.75$, 95% CI[0.68, 0.80]), fatigue ($r_s = 0.77$, 95% CI[0.72, 0.80]), functional impairment ($r_s = 0.79$, 95% CI[0.75, 0.83]), and general health ($r_s = -0.58$, 95% CI[−0.65, −0.50]), all at $p < 0.001$.

The sequential regression analyses demonstrated that depression and anxiety significantly predicted functional impairment ($R^2 = 0.377$, 95% CI[0.30, 0.46], $p < 0.001$). Addition of the GSQ-30 total score resulted in a significant ΔR^2 of 0.244 (95% CI[0.17, 0.32], $p < 0.001$), indicating that the GSQ-30 total predicted functional impairment over and above symptoms of anxiety/depression. Further, when the GSQ-30 was added as the first predictor, it accounted for 57% (95% CI[0.50, 0.63]) of the variance in functional impairment with depression/anxiety contributing only an additional 5% (95% CI[0.02, 0.09]) when added later.

The Welch's one-way test with *post-hoc* comparisons indicated a significant difference between all health status groups on the GSQ-30 [$F_{(4, 95.28)} = 74.96$, $p < 0.001$]. GSQ-30 mean scores increased stepwise with each categorical decrease in reported general health ("excellent"($M = 13.49$, $SD = 14.2$); "very good"($M = 20.73$, $SD = 19.7$); "good"($M = 34.67$, $SD = 20.95$); "fair"($M = 47.82$, $SD = 20.81$); "poor"($M = 71.22$, $SD = 13.05$); p-values ranged between <0.001 and 0.01.

TABLE 2 | Summary of factor loadings for all clinical cases ($N = 305$).

	NeuroΨ symptoms	Neurological symptoms	Pain/fatigue symptoms	Viral-like symptoms
Slower speed of thinking	0.89			
Trouble with memory	0.88			
Trouble finding words or retrieving names	0.88			
Feeling panicky, anxious or worried	0.82			
Feeling irritable, sad, or decreased pleasure	0.81			
Hot or cold sensations in extremities		0.70		
Balance problems or sense of room-spinning		0.69		
Bladder discomfort or change in urination		0.65		
Numbness or tingling		0.64		
Skin or muscle twitching		0.59		
Change in visual clarity or trouble focusing		0.56		
Light-headed or uncomfortable on standing		0.53		
Irregular or rapid heart beats		0.53		
Discomfort with normal light or sound		0.52		
Shooting, stabbing or burning pains		0.50	0.43	
Shortness of breath		0.45		
Muscle aches or pains			0.92	
Joint pain or swelling			0.87	
Muscle weakness			0.75	
Back pain			0.66	
Feeling worse after normal physical exertion			0.58	
Stiff or painful neck			0.58	
Feeling fatigued or having low energy	0.40		0.51	
Not feeling rested on awakening	0.44		0.46	
Feeling feverish				0.99
Sweats and/or chills				0.85
Headaches				0.58
Nausea and/or vomiting				0.55

Loadings of <0.40 are not displayed.

There was a statistically significant difference between EM, PTLDS, depression, TBI, and healthy controls as determined by Welch's one-way test [$F_{(4, 128.69)} = 77.79$, $p < 0.001$]. *Post-hoc* tests indicated significant differences (all p-values < 0.001) on the GSQ-30 total score between the healthy control group ($M = 6$, $SD = 7.37$), and all other groups: EM ($M = 24.15$, $SD = 20.11$), PTLDS ($M = 42.38$, $SD = 22.14$), depressed ($M = 42.28$, $SD = 21.05$) and TBI ($M = 32.82$, $SD = 26.79$). The GSQ-30 total score for the EM group was also significantly different from PTLDS and depression. No other comparisons were significant.

Factorial Validity

PCA was conducted with data from all participants except healthy controls. The Kaiser-Meyer-Olkin measure verified the sampling adequacy for the analysis, KMO = 0.86, indicating a "meritous" degree of common variance (28, 29) and Bartlett's test of sphericity was significant ($p < 0.001$), indicating sufficiently large correlations between items for PCA. Initial examination of eigenvalues revealed five components with eigenvalues >1. The parallel test and the scree plot suggested a more conservative 4-component solution. Given convergence of the scree plot and the parallel test, and the tendency for Kaiser's criterion to overestimate the true number of components (30), a 4-component solution was examined.

Items with component loadings ≥0.40 were retained. After the first rotation, two items ("trouble falling or staying asleep" and "needing more sleep than usual") were removed due to loadings <0.40. The final solution accounted for 65.11% of variance. The item clusters suggested that component 1 represented neuropsychiatric problems and explained 17.11% of the variance, component 2 represented neurological symptoms and explained 18.93%, component 3 represented pain and fatigue symptoms and explained 16.93%, and component 4 represented viral-like symptoms and explained 12.14%. See **Table 2** for component loadings. Cronbach's alpha for the GSQ-30 total score did not change substantially as a result of removing the two items, $r = 0.94$. The PCA was also run using only the Lyme participants (EM and PTLDS) as a supplementary analysis to examine factorial invariance. A similar structure emerged except: (a) the two sleep items were retained within the pain/fatigue component; and (b) the item 'light-headed or uncomfortable on standing'

TABLE 3 | Summary of factor loadings for EM and PTLDS ($N = 218$).

	Pain/fatigue symptoms	Neuropsychiatric symptoms	Neurological symptoms	Viral-like symptoms
Muscle aches or pains	0.92			
Joint pain or swelling	0.76			
Muscle weakness	0.76			
Stiff or painful neck	0.65			
Back pain	0.65			
Feeling worse after normal physical exertion	0.6			
Not feeling rested on awakening	0.56			
Feeling fatigued or having low energy	0.54	0.43		
Needing more sleep than usual	0.49			
Trouble falling or staying asleep	0.48			
Slower speed of thinking		0.85		
Trouble with memory		0.84		
Trouble finding words or retrieving names		0.84		
Feeling panicky, anxious or worried		0.81		
Feeling irritable, sad, or decreased pleasure		0.74		
Hot or cold sensations in extremities			0.64	
Skin or muscle twitching			0.63	
Numbness or tingling			0.58	
Bladder discomfort or change in urination			0.55	
Balance problems or sense of room-spinning			0.54	
Change in visual clarity or trouble focusing			0.51	
Shortness of breath			0.5	
Irregular or rapid heart beats			0.5	
Shooting, stabbing or burning pains			0.45	
Discomfort with normal light or sound			0.43	
Feeling feverish				0.91
Sweats and/or chills				0.75
Headaches				0.54
Nausea and/or vomiting				0.5
Light-headed or uncomfortable on standing				0.43

Loadings of <0.40 are not displayed.

loaded on the viral-like symptoms component. See **Table 3** for component loadings of the PCA for the Lyme sample. Due to the presence of a Heywood case, the solution reported used an "Oblimin" rotation rather than "Promax."

Sensitivity to Change

Among the 53 patients with EM who had baseline and 6 month ratings, GSQ-30 total scores decreased significantly from baseline ($M = 21.93$, $SD = 20.60$) to 6 months post-treatment ($M = 13.06$, $SD = 15.56$; $p < 0.01$), with median and mean percent change in symptoms over the 6 month period of 51.87% (IQR = 1.44–89.29%) and 16.69%(SD = 102.38), respectively. The range (−300% to 100%) included participants with deterioration and improvement of symptoms over time. The percentage change over time for the three outcome groups differed as expected: Returned to health ($Md = +80.68\%/M = +50.55\%$), Symptoms only ($Md = +24.59/M = -38.55\%$), PTLDS ($Md = -16.56\%/M = -38.56\%$).

While most of the 53 patients with EM recovered fully ($n = 34$), others had symptoms without functional impairment ($n = 11$) and a smaller group had symptoms with functional impairment (PTLDS) ($n = 8$). There was a statistically significant difference in GSQ-total score between outcome groups at 6 months [$F_{(2,11.52)} = 20.68$, $p < 0.001$]. *Post-hoc* tests revealed that all pairwise outcome group comparisons were significantly different; see **Figure 3**. Return to health ($M = 4.65$, $SD = 4.68$) was significantly different from both Symptoms only ($p = 0.006$) and PTLDS ($p = 0.003$). PTLDS ($M = 37.38$, $SD = 16.77$) was different from Symptoms only ($M = 21.36$, $SD = 14.14$) ($p = 0.046$).

Results from the mixed ANOVA indicated that there was a significant main effect of outcome group on the GSQ total score [$F_{(2,50)} = 20.36$, $p < 0.001$, generalized $\eta^2 = 0.32$], and time [$F_{(1,50)} = 10.81$, $p < 0.01$, generalized $\eta^2 = 0.08$], but that there was no interaction between group and time [$F_{(2,50)} = 1.29$, $p = 0.28$, generalized $\eta^2 = 0.02$]; this indicates that change in GSQ total score from baseline over time was not significantly different between outcome groups. Change in GSQ-30 total score including all 53 EM patients from baseline to 6 month follow-up was significantly correlated with change in functional impairment ($r_\tau = 0.61$, $p < 0.001$).

Exploratory Analyses With Factor-Derived Subscales

Welch's one-way tests indicated significant differences between clinical groups across three of four subscales; pain and fatigue [$F_{(3,114.86)} = 17.04$, $p < 0.001$], neuropsychiatric symptoms [$F_{(3,110.45)} = 36.19$, $p < 0.001$], and neurological symptoms [$F_{(3,108.29)} = 11.96$, $p < 0.001$]. Pairwise *post-hoc* comparisons (**Figure 2**) revealed significant differences between PTLDS and both EM and TBI on the pain and fatigue subscale; and between PTLDS and EM on both the neurologic and the neuropsychiatric subscales. All other comparisons were not significant.

Using the factor-derived subscales, paired samples *t*-tests were conducted to examine change over time in the EM group for participants with available 6 month follow-up data ($n = 53$). Results demonstrated a significant reduction in pain/fatigue and viral-like symptoms for patients with a documented EM rash (see **Table 4**).

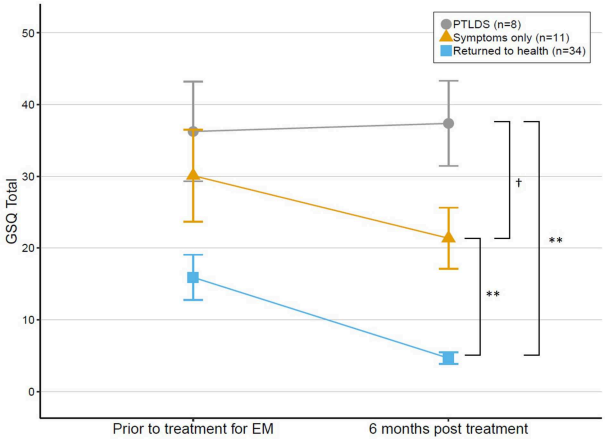

FIGURE 3 | Change in GSQ-30 scores among patients treated for EM at baseline and reassessed 6 months later, grouped by outcome status at 6 months. Error bars represent standard errors. $^{\dagger}p = 0.05$, $^{**}p < 0.01$.

DISCUSSION

The GSQ-30 is a psychometrically sound measure of symptom burden among patients with multi-system illness. It has the advantages of brevity, ease of administration and scoring, and sensitivity to change after treatment. Using a multi-site cohort of 342 participants, the GSQ-30 demonstrated excellent internal consistency among items. Not surprisingly, the GSQ-30 was significantly associated with other construct-related measures such as brief scales of depression, anxiety, fatigue, pain, and general health. That the GSQ-30 is not simply another way of assessing anxiety or depression was supported by the regression analysis which indicated that the GSQ-30 accounted for an additional 25% of variance in the functional impairment score beyond that contributed by anxiety and depression.

Notably, the GSQ-30 total score correlated strongly with functional impairment. In addition, reduction in the GSQ-30 over time corresponded with improvement in functional status. These findings support the conclusion that the GSQ-30 detects symptom impact (i.e., burden) and not just presence.

The factor analysis led to the identification of four core domains: viral-like, pain/fatigue, neurologic, and neuropsychiatric symptoms. These are common symptom clusters reported by patients impacted by Lyme disease. The domain profile of PTLDS differed from that of EM, with the former having a significantly greater burden of pain/fatigue, neuropsychiatric, and neurologic symptoms. These results, as well as the finding that the GSQ-30 total score for PTLDS was nearly 2x higher than for EM, support the clinical impression that patients with PTLDS have a much greater symptom burden than those with early Lyme disease.

The analysis of change among patients with EM after antibiotic treatment identified significant improvement over time in both total score as well as in subscales of pain/fatigue and viral-like symptoms. That significant improvement was not seen in the

TABLE 4 | *t*-tests with factor-derived subscales and total GSQ scores for antibiotic-treated EM cases.

Scale	Baseline *M* (SD)	6-months *M* (SD)	*t*	*P* value	Cohen's *d*
Pain/Fatigue	1.09 (0.96)	0.70 (0.79)	2.92	<0.01	0.45
Neuropsychiatric	0.54 (0.75)	0.51 (0.68)	0.34	0.74	0.05
Neurological symptoms	0.37 (0.53)	0.25 (0.45)	1.95	0.06	0.25
Viral-like symptoms	0.96 (0.98)	0.26 (0.43)	4.94	<0.001	0.91
Total GSQ (summed)	21.93 (20.60)	13.06 (15.56)	3.27	<0.001	0.49

subscales of neurologic and neuropsychiatric symptoms raises several questions. Are these domains reflective of symptoms triggered by infection but not due to persistent infection? Is a different antibiotic or another mode of treatment (e.g., anti-inflammatory or neuromodulatory interventions) needed to reduce symptoms in these domains? Can the subscale scores on the GSQ-30 be used to guide treatment planning? These clinically important questions can be addressed in future research.

This study demonstrated that the research algorithms used to categorize patients' treatment response as "Returned to health," "Symptoms only," or "PTLDS" correspond with scores on the GSQ-30. The "Returned to health" group had significantly lower GSQ-30 total scores (mean 6 and 4.65, respectively) compared to the Symptoms only group (mean 21.36) which in turn had significantly lower scores than the PTLDS group (mean 37.38). Poor outcome at 6 months may be due to many causes, including persistent infection, post-infectious processes or re-infection. Regular administration of the GSQ-30 may improve outcome by highlighting for the clinician the symptoms of greatest burden to the patient which may need a different treatment approach. Strikingly, the GSQ-30 total score for the EM patients who developed PTLDS on average was high at the first assessment and remained high at the 6 month assessment after treatment, while the recovered group had markedly lower scores at baseline which declined with treatment (**Figure 3**). This raises the possibility that the magnitude of the GSQ-30 at initial evaluation may identify a subgroup of EM patients in need of treatment augmentation to increase the likelihood of improved long-term outcome. Whether the GSQ-30 total score corresponds with particular biomarkers, such as inflammatory cytokines, would be of great interest for future exploration. The GSQ-30 therefore appears to be a useful instrument to complement clinical judgment and ratings of symptom burden.

We examined how patients with PTLDS compared to those with depression and TBI on the GSQ-30 total and subscale scores. The lack of a significant difference in total scores may reflect the multi-system involvement in all three disorders. This highlights that the GSQ-30 is a measure of symptom burden and not a diagnostic instrument. The subscales however may reveal symptom profiles that differ between disorders, as in the contrast between PTLDS and TBI on the pain/fatigue subscale. The lack of difference between PTLDS and depression in both total and subscale scores highlights the striking overlap between these two disorders. Both are associated with disturbances of energy, sleep, cognition, pain, and mood and both may be mediated by common central nervous system immune mechanisms (31, 32).

The strengths of this study include the large sample size, the selection of items common to patients with early and post-treatment Lyme disease syndrome, the identification of subscales statistically that have clinical face validity, and the demonstration of significant change using prospectively collected data among patients with EM before and after standardized treatment. The primary limitation of this study is that we could not assess sensitivity to change of the GSQ-30 in the PTLDS group, as we did not have access to a prospectively treated group of PTLDS patients before and after treatment. PTLDS is a more heterogeneous condition than EM; this may impact the ability of the GSQ-30 to assess change over time. A second limitation is that although the healthy control group was required to be seronegative for *B. burgdorferi* antibodies, the TBI and depressed patients were not serologically tested; therefore, we cannot rule-out prior unrecognized infection with *B. burgdorferi* in some of the latter patients.

Future studies should examine the usefulness of the GSQ-30 in other infected cohorts (e.g., *Babesia microti, Borrelia miyamotoi*), the relationship of the GSQ-30 to specific biomarkers, and whether clinical outcome can be improved by using the GSQ-30 to guide treatment reassessment.

In conclusion, the GSQ-30 is a valid and reliable instrument to assess symptom burden among patients with acute and post-treatment Lyme disease syndrome and is sensitive in the detection of change after antibiotic treatment among individuals with EM.

DATA AVAILABILITY STATEMENT

Participant consent did not include seeking permission for data to be made publicly available.

ETHICS STATEMENT

This study, involving human participants, was reviewed and approved by each institution's respective IRB (The New York State Psychiatric Institute, Johns Hopkins University School of Medicine, Partners Health Care). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BA and JA had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. CB and JM conducted the statistical analyses.

BF, SD, NZ, and JA take responsibility for the study's concept and design. BF, CB, SD, JA, and AR participated in the drafting of the manuscript. BF obtained funding. SD, AR, and NO provided administrative, technical, or material support. BF, JA, and NZ provided supervision. All authors contributed to the acquisition, analysis or interpretation of the data and all contributed to critical revision of the manuscript for important intellectual content.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Evaluation of Natural and Botanical Medicines for Activity Against Growing and Non-growing Forms of *B. burgdorferi*

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Lyme disease is the most common vector-borne disease in the US and Europe. Although the current recommended Lyme antibiotic treatment is effective for the majority of Lyme disease patients, about 10–20% of patients continue to suffer from persisting symptoms. There have been various anecdotal reports on the use of herbal extracts for treating patients with persisting symptoms with varying degree of improvements. However, it is unclear whether the effect of the herb products is due to their direct antimicrobial activity or their effect on host immune system. In the present study, we investigated the antimicrobial effects of 12 commonly used botanical medicines and three other natural antimicrobial agents for potential anti-*Borrelia burgdorferi* activity *in vitro*. Among them, 7 natural product extracts at 1% were found to have good activity against the stationary phase *B. burgdorferi* culture compared to the control antibiotics doxycycline and cefuroxime. These active botanicals include *Cryptolepis sanguinolenta*, *Juglans nigra* (Black walnut), *Polygonum cuspidatum* (Japanese knotweed), *Artemisia annua* (Sweet wormwood), *Uncaria tomentosa* (Cat's claw), *Cistus incanus*, and *Scutellaria baicalensis* (Chinese skullcap). In contrast, *Stevia rebaudiana*, *Andrographis paniculata*, Grapefruit seed extract, colloidal silver, monolaurin, and antimicrobial peptide LL37 had little or no activity against stationary phase *B. burgdorferi*. The minimum inhibitory concentration (MIC) values of *Artemisia annua*, *Juglans nigra*, and *Uncaria tomentosa* were quite high for growing *B. burgdorferi*, despite their strong activity against the non-growing stationary phase *B. burgdorferi*. On the other hand, the top two active herbs, *Cryptolepis sanguinolenta* and *Polygonum cuspidatum,* showed strong activity against both growing *B. burgdorferi* (MIC = 0.03–0.06% and 0.25–0.5%, respectively) and non-growing stationary phase *B. burgdorferi.* In subculture studies, only 1% *Cryptolepis sanguinolenta* extract caused complete eradication, while doxycycline and cefuroxime and other active herbs could not eradicate *B. burgdorferi* stationary phase cells as many spirochetes were visible after 21-day subculture. Further studies are needed to identify the active constituents of the effective botanicals and evaluate their combinations for more effective eradication of *B. burgdorferi in vitro* and *in vivo*. The implications of these findings for improving treatment of persistent Lyme disease are discussed.

Keywords: *Borrelia burgdorferi*, Lyme disease, persisters, botanical medicines, herbs, natural medicines, antimicrobial activity, biofilm

INTRODUCTION

Lyme disease, caused by *Borrelia burgdorferi,* and multiple closely related *Borrelia* species, is the most common vector-borne human disease in the Northern Hemisphere (1, 2). About 300,000 new cases are diagnosed in the United States annually (3, 4). Tick-borne infections are on the rise in the USA and Europe due to a host of different factors including climate change (5, 6) and disruption of predator density in suburban areas (7). Recent studies on tick prevalence and pathogen load have identified new geographical areas where vector ticks are present (8), as well as novel tick-borne pathogens present in areas where they had not previously been identified (such as *B. miyamotoi* in Northern California) (9).

Lyme disease can affect many different body systems and organs (10). While many patients recover fully with early antibiotic therapy, at least 10–20% of patients experience persistent symptoms following the conventionally recommended course of 2–4 weeks of antibiotics (11, 12), and a recent retrospective analysis documented 63% of patients experienced persistent symptoms after receiving antibiotic treatment for Lyme disease (13). Patients who experience persistent symptoms can have significant and ongoing disability (11, 14) and increased health care costs and utilization (13). *B. burgdorferi* can evade the immune system response (15, 16) and multiple studies have shown that the bacteria is capable of persisting in diverse tissues across a variety of animal models despite aggressive and prolonged antibiotic therapy (17–19).

In addition to the mammalian studies noted above, *B. burgdorferi* persistence following antibiotic treatment has been demonstrated in human studies and case reports (20–23). Persistent Lyme borreliosis symptoms significantly affect quality of life (24, 25), therefore some physicians treat these patients with extended courses of antibiotics. However, this approach is controversial with one medical society guideline (26) advocating against retreating patients with persistent (>6 months) symptoms and another medical society guideline (27) recommending individualized risk-benefit assessments and potential retreatment or longer duration treatment of patients with persistent symptoms. While antibiotic retreatment has been associated with improved clinical outcomes (27, 28), antibiotic therapy appears to be more effective against the actively dividing spirochete form. In addition, it has been shown that *B. burgdorferi* can change morphology and form biofilm-like microcolonies consisting of stationary phase persister bacteria (29–31). Traditional antibiotics have poor activity against the atypical persister forms (round bodies, microcolonies, and biofilm) and we have previously worked to identify novel drugs and drug combinations that are effective against these atypical forms (29, 30, 32). While Daptomycin and Dapsone have been identified as having significant effects against borrelia persister cells *in vitro* (29, 33) and *in vivo* in a murine model (31), their use in clinical practice can be limited by side effects (both), cost (daptomycin), parenteral administration (daptomycin), and poor CNS penetration (daptomycin) (34). Given the limitations of current Lyme treatment it is of vital importance that novel, safe, and effective therapies be identified for clinical use.

Importantly, botanical medicines have been shown to have *in vitro* antimicrobial activity against various morphologic forms of *B. burgdorferi*. Because there are a limited number of studies evaluating the effects of botanical medicine on *B. burgdorferi*, it is helpful to draw on clinical studies that have shown benefit using botanical medicines for other spirochetal infections and infections like mycobacterium that are known to form antibiotic tolerant persister cells (35). For example, *Andrographis* has been shown to effectively treat leptospirosis in Chinese clinical trials (36) and improve clinical outcomes when combined with standard treatment for tuberculosis (37).

Botanical medicine has a long history of use, beginning almost 5,000 years ago in Mesopotamia and has over 3,000 years of documented usage in China (38). The safety of botanical medicines has been documented in various traditional systems of medicine such as Ayurvedic Medicine and Traditional Chinese Medicine over centuries. Recent retrospective and systematic reviews in the European Union and South America have concluded severe adverse events associated with Botanical Medicine usage were rare (39, 40).

This study builds on previous studies that used our *in vitro* stationary phase persister model and SYBR Green I/propidium iodide (PI) assay to screen potential antimicrobial candidates. Having previously identified novel drugs and drug combinations from an FDA drug library (32), as well as selected botanicals in essential oil form that have anti-*B. burgdorferi* activity (41, 42), in the present study (Feng et al. https://www.biorxiv.org/content/10.1101/652057v1.full), we investigated the effect of 12 botanical medicines and 3 other natural antimicrobial agents for potential anti-*B. burgdorferi* activity *in vitro*.

MATERIALS AND METHODS

Strain, Media, and Culture Techniques

B. burgdorferi strain B31 was cultured in BSK-H medium (HiMedia Laboratories Pvt. Ltd.) with 6% rabbit serum (Sigma-Aldrich, St. Louis, MO, USA). All culture medium was filter-sterilized by 0.2 μm filter. Cultures were incubated in sterile 50 ml conical tubes (BD Biosciences, CA, USA) in microaerophilic incubator (33°C, 5% CO_2) without antibiotics.

Botanical and Natural Medicines

A panel of natural product extracts: *Polygonum cuspidatum*, *Cryptolepis sanguinolenta*, *Artemisia annua*, *Juglans nigra*, *Uncaria tomentosa, Scutellaria baicalensis*, *Stevia rebaudiana*, *Cistus incanus*, *Andrographis paniculata*, *Ashwagandha somnifera*, *Dipsacus fullonum rad,* grapefruit seed extract, LL37, monolaurin, colloidal silver, and relevant solvent controls (see **Table 1**) were identified. The botanical medicines or natural products were chosen based on anecdotal clinical usage and preclinical data from the literature. Primary criteria for selecting compounds for the present study included agents that had shown significant anti-borrelial effects in previous studies, have favorable safety profiles and can be absorbed systemically. Additional criteria for selecting

TABLE 1 | Botanical and natural medicine sources, validation, and testing.

Natural product	Source	Validation/ID	Contamination	Details
Citrus x paradisi	Cintamani, Poland (Citrosept™)	Cintamani, Poland	<1 ppm for Benzalkonium chloride, Triclosan, Benzoic Acid	Organic grapefruit seed extract
Stevia rebaudiana	Sonoma County Herb Exchange (cultivated)	Organoleptic, KW Botanicals	Not tested	25% ETOH extract by KW Botanicals
Juglans nigra	Pacific Botanicals (wild harvested)	Organoleptic, KW Botanicals	Not tested	45% ETOH extract of husk/hulls by KW Botanicals
Dipsacus fullonum	Friend's of the Trees (wild harvested, Washington State)	DNA species identification, NSF International	Not tested	40% ETOH by KW Botanicals (inadvertently co-mingled with *D. asper* sample prior to testing)
Dipsacus asper	KW Botanicals (wild harvested, California)	DNA species identification, NSF International	Not tested	40% ETOH by KW Botanicals (inadvertently co-mingled with D. fullonum sample prior to testing)
Uncaria tomentosa	Mountain Rose Herbs (wild harvested)	DNA species identification, Christopher Hobbs, Ph.D.	Negative testing for aerobic plate count, *E. coli*, coliform, salmonella, yeast & mold	50% ETOH by KW Botanicals
Artemisia annua	Heron Botanicals (organic cultivation)	American Herbal Pharmacopeia (Scotts Valley, CA), Organoleptic, Heron Botanicals Confirmed 0.11% Artemisinin content, The Institute for Food Safety and Defense	Negative testing for aerobic plate count and yeast & mold	30, 60, and 90% ETOH by Heron Botanicals
Withania somnifera	Heron Botanicals (organic cultivation)	HPTLC, The Institute for Food Safety and Defense Organoleptic, Heron Botanicals	Negative testing for Pb, Cd, Hg, As, aerobic plate count, and yeast & mold	30, 60, and 90% ETOH by Heron Botanicals
Juglans nigra	Heron Botanicals (wild harvested, New York)	Organoleptic, Heron Botanicals	Positive aerobic plate count: 960 CFU/ml (acceptable limit 1,000 CFU/ml) negative testing for Pb, Cd, Hg, As, and yeast & mold	30, 60, and 90% ETOH by Heron Botanicals
Andrographis paniculata	Heron Botanicals (organic cultivation, China)	Organoleptic, Heron Botanicals	Negative testing for pesticides, sulfur dioxide, aerobic plate count, and yeast & mold	30, 60, and 90% ETOH by Heron Botanicals
Polygonum cuspidatum	Heron Botanicals (organic cultivation, China)	Organoleptic, Heron Botanicals	Negative testing for pesticides, sulfur dioxide, aerobic plate count, and yeast & mold	30, 60, and 90% ETOH by Heron Botanicals
Scutellaria baicalensis	Heron Botanicals (organic cultivation, China)	Organoleptic, Heron Botanicals	Negative testing for pesticides, sulfur dioxide, aerobic plate count, and yeast & mold	30, 60, and 90% ETOH by Heron Botanicals
Cryptolepis sanguinolenta	Heron Botanicals (wild harvested, Ghana)	HPTLC, The Institute for Food Safety and Defense Organoleptic, Heron Botanicals	Negative testing for Pb, Cd, Hg, As, aerobic plate count, and yeast & mold	30, 60, and 90% ETOH by Heron Botanicals
Cistus incanus	BioPure Healing Products™	DNA species identification, NSF International	Negative testing for aerobic plate count, *E. coli*, coliforms, and yeast & mold	45% ETOH by BioPure Healing Products (aerial parts). DNA analysis reports Cistus Incanus and *Cistus albidus* are genetically indistinguishable
Monolaurin	Lauricidin™	Per manufacturer	Not tested	Dissolved in 100% DMSO
Colloidal silver	Argentyn 23™	Per manufacturer	Not tested	No control available
LL37	Taylor Made Pharmacy	Per manufacturer	Not tested	LL37 and control solution provided by Taylor Made Pharmacy

compounds included anecdotal reports from patients and/or providers, anti-biofilm effects and ability to cross the blood brain barrier.

Botanical medicines were sourced from KW Botanicals (San Anselmo, California) and Heron Botanicals (Kingston, Washington). Botanicals were identified via macroscopic and organoleptic methods and voucher specimens are on file with the respective production facilities. Most botanical medicines were provided as alcohol extracts at 30, 60, and 90% alcohol, and the alcohol used was also tested separately as a control in different dilutions. Monolaurin (Lauricidin™ brand) (dissolved in 100% DMSO), and colloidal silver (Argentyn™ brand) were purchased commercially. LL37 and a control was obtained from Taylor Made Pharmacy in Nicholasville, KY. Citrosept™ (Cintamani, Poland) and Nutribiotic™ grapefruit seed extract products and a control were purchased commercially. See **Table 1** for additional details on sourcing, testing, and validation of botanical and natural medicines used.

Doxycycline (Dox) and cefuroxime (CefU) (Sigma-Aldrich, USA) were dissolved in suitable solvents (43) to form 5 mg/ml stock solutions. The antibiotic stocks were filter-sterilized by 0.2 μm filter and stored at −20°C.

Microscopy

B. burgdorferi spirochetes and aggregated microcolonies treated with natural products or control drugs were stained with SYBR Green I and PI (propidium iodide) and checked with BZ-X710 All-in-One fluorescence microscope (KEYENCE, Itasca, IL, USA). The bacterial viability was performed by calculating the ratio of green/red fluorescence to determine the ratio of live and dead cells, as described previously (29). The residual cell viability reading was obtained by analyzing three representative images of the same bacterial cell suspension taken by fluorescence microscopy. To quantitatively determine the bacterial viability from microscope images, Image Pro-Plus software was employed to evaluate fluorescence intensity as described previously (30).

Evaluation of Natural Products for Their Activity Against *B. burgdorferi* Stationary Phase Cultures

B. burgdorferi B31 was cultured for 7 days in microaerophilic incubator (33°C, 5% CO_2) as stationary phase cultures ($\sim 10^{7-8}$ spirochetes/mL). To evaluate potential anti-persister activity of the natural products, their stocks and their control solvents were added to 100 μL of the *B. burgdorferi* stationary phase culture in 96-well plates to obtain the desired concentrations. The botanical medicines and natural product extracts were tested with the concentration of 1, 0.5, and 0.25% (v/v); antibiotics of daptomycin, doxycycline, and cefuroxime were used as controls at a final concentration of 5 μg/ml. All the tests mentioned above were run in triplicate. The microtiter plates were sealed and incubated at 33°C without shaking for 7 days with 5% CO_2.

Subculture Studies to Confirm the Activity of the Top Natural Product Hits

For the subculture study, 1 mL *B. burgdorferi* stationary phase culture was treated by natural products or control drugs in 1.5 ml Eppendorf tubes for 7 days at 33°C without shaking. Next, cells were centrifuged, and cell pellets were washed with fresh BSK-H medium (1 mL) followed by resuspension in fresh BSK-H medium without antibiotics. Then 50 μl of cell suspension was inoculated into 1 ml of fresh BSK-H medium for subculture at 33°C, 5% CO_2. Cell growth was monitored using SYBR Green I/PI assay and fluorescence microscopy after 7–20 days.

RESULTS

Evaluation of Activity of Natural Product Extracts Against Stationary Phase *B. burgdorferi*

We tested a panel of botanical medicines and natural product extracts and their corresponding controls against a 7-day old *B. burgdorferi* stationary phase culture in 96-well plates incubated for 7 days. **Table 2** summarizes the activity of these natural product extracts against the stationary phase *B. burgdorferi* culture at 1, 0.5, and 0.25%. Among them, 7 natural product extracts at 1% were found to have strong activity against the stationary phase *B. burgdorferi* culture compared to the control antibiotics doxycycline and cefuroxime (**Table 2**). To eliminate auto-fluorescence background, we checked the ratio of residual live cells and dead cells by examining microscope images as described previously (30). Using fluorescence microscopy, we confirmed that 1% *Cryptolepis sanguinolenta*, *Juglans nigra*, and *Polygonum cuspidatum* could eradicate almost all live cells with only dead and aggregated cells left as shown in **Figure 1**. At 0.5% concentration, 11 natural product extracts (*Polygonum cuspidatum* 60% EE, *Cryptolepis sanguinolenta* 60% EE, *Artemisia annua* 90% EE, *Juglans nigra* 30–60% EE, *Uncaria tomentosa* WE, *Artemisia annua* 60% EE, *Polygonum cuspidatum* 90% EE, *Scutellaria baicalensis*) still exhibited stronger activity than the current clinically used doxycycline and cefuroxime (**Table 2** and **Figure 1**). Among them, the most active natural product extracts were *Cryptolepis sanguinolenta* 60% EE, *Polygonum cuspidatum* 60% EE, *Artemisia annua* 90% EE, *Juglans nigra* 60% EE, *Uncaria tomentosa* WE, *Artemisia annua* 60% EE, because of their outstanding activity even at 0.25%, as shown by better activity than control drugs (**Table 2** and **Figure 1**). In particular, 0.25% *Cryptolepis sanguinolenta* could eradicate or dissolve all the *B. burgdorferi* cells including aggregated forms as we found rare live and even dead cells with SYBR Green I/PI microscope observation (**Figure 1**). Although *Juglans nigra* could eradicate almost all stationary phase *B. burgdorferi* cells at 0.5% (**Figure 1**), it could not kill the aggregated microcolony form at 0.25% as shown by many live (green) microcolonies by SYBR Green I/PI microscopy. Although the plate reader data showed *Polygonum cuspidatum* 60% ethanol extract had the strongest activity at 0.25%, the microscope result did not confirm it due to higher residual viability than that of *Cryptolepis sanguinolenta* and *Juglans nigra* (**Figure 1**).

We also tested several other herbs and substances that are used by Lyme patients including *Stevia rebaudiana*, *Andrographis paniculata*, Grapefruit seed extract, *Ashwagandha somnifera*, Colloidal silver, Lauricidin, and antimicrobial peptide LL-37, but found they had little or no activity against stationary phase *B. burgdorferi* cells.

MIC Values of the Active Natural Product Extracts

Because the activity of antibiotics against non-growing *B. burgdorferi* is not always correlated with their activity against growing bacteria (30), we therefore determined the MICs of these natural product extracts against the replicating *B. burgdorferi* as described previously (32). The MIC values of some natural product extracts such as *Artemisia annua*, *Juglans nigra*, *Uncaria tomentosa* were quite high for growing *B. burgdorferi*, despite their strong activity against the non-growing stationary phase *B. burgdorferi* cells (**Table 2**). On the other hand, the top two active natural product extracts *Cryptolepis sanguinolenta* and *Polygonum cuspidatum* showed strong activity against the growing *B. burgdorferi* with a low MIC (0.03–0.06% and 0.25–0.5% respectively) and also non-growing stationary phase *B. burgdorferi* (**Table 2**).

TABLE 2 | Activity of natural products against growing (MIC) and stationary phase *B. burgdorferi*.

Natural products	MIC (%)[a]	Stationary phase residual viability (%) at different concentrations of herbs[b]			Subculture	
		1%	0.5%	0.25%	1%	0.5%
Drug free control			94%		+	
5 μg/ml Doxycycline	0.25 μg/mL		74%		+	
5 μg/ml Cefuroxime	0.13 μg/mL		65%		+	
30% alcohol control	>2%	79%	80%	95%	+	+
60% alcohol control	1–2%	77%	76%	94%	+	+
90% alcohol control	0.5–1%	75%	79%	91%	+	+
Polygonum cuspidatum 60% EE	**0.25–0.5%**	**30%**	**41%**	**43%**	+	+
Cryptolepis sanguinolenta 60% EE	**0.03–0.06%**	**46%**	**48%**	**46%**	–	+[c]
Artemisia annua 90% EE	0.5–1%	**43%**	**50%**	**49%**	+	+
Juglans nigra 60% EE	0.5–1%	**14%**	**36%**	**53%**	+	+
Uncaria tomentosa (inner bark) WE	1–2%	**49%**	**47%**	**54%**	+	+
Polygonum cuspidatum 90% EE	**0.25–0.5%**	**21%**	**43%**	**61%**	+	+
Juglans nigra 30% EE	1–2%	**33%**	**50%**	**62%**	+	+
Artemisia annua 60% EE	0.5%–1%	**44%**	**44%**	**55%**	+	+
Scutellaria baicalensis	>2%	**59%**	**60%**	**62%**	+	+
Cryptolepis sanguinolenta 90% EE	**0.03–0.06%**	**48%**	**47%**	**63%**	ND	ND
Juglans nigra 90% EE	0.5–1%	**34%**	**56%**	**63%**	ND	ND
Cryptolepis sanguinolenta 30% EE[d]	**0.06–0.13%**	**59%**	**64%**	**63%**	ND	ND
Juglans nigra fruc	1–2%	**52%**	**59%**	66%	ND	ND
Scutellaria baicalensis 60% EE	**0.25–0.5%**	**62%**	67%	67%	ND	ND
Scutellaria baicalensis 90% EE	**0.25–0.5%**	72%	74%	75%	ND	ND
Andrographis paniculata 90% EE	0.5–1%	74%	75%	75%	ND	ND
Scutellaria baicalensis 30% EE	**0.25–0.5%**	80%	72%	77%	ND	ND
Cistus incanus	**0.25–0.5%**	**29%**	74%	77%	ND	ND
Andrographis paniculata 30% EE	1–2%	79%	78%	78%	ND	ND
Chuan Xin Lian	>2%	89%	86%	85%	ND	ND
Citrosept™	1–2%	89%	90%	85%	ND	ND
Polygonum cuspidatum 30% EE[d]	**0.25–0.5%**	**34%**	65%	87%	ND	ND
Lauricidin™	>2%	88%	86%	87%	ND	ND
Scutellaria barbata	>2%	**58%**	**60%**	88%	ND	ND
Stevia rebaudiana fol	>2%	86%	66%	88%	ND	ND
Andrographis paniculata 60% EE	1–2%	76%	77%	88%	ND	ND
Dipsacus fullonum rad	>2%	84%	90%	89%	ND	ND
LL37 antimicrobial peptide	>2%	91%	91%	89%	ND	ND
Uncaria tomentosa	>2%	68%	90%	91%	ND	ND
Ashwagandha somnifera 90% EE	0.5–1%	76%	76%	92%	ND	ND
Ashwagandha somnifera 60% EE	0.5–1%	79%	81%	92%	ND	ND
Colloidal silver (Argentyn™)	>2%	88%	85%	92%	ND	ND
Ashwagandha somnifera 30% EE	0.5–1%	94%	94%	93%	ND	ND
Citrosept™	1–2%	98%	99%	95%	ND	ND
Grapefruit seed extract	Citrus paradisi	78%	81%	94%	ND	ND

[a] *The standard microdilution method was used to determine the minimum inhibitory concentration (MIC). The MICs below 0.5% are shown in bold.*

[b] *A 7-day old B. burgdorferi stationary phase culture was treated with natural product extracts or control drugs for 7 days. Bold type indicates the samples that had better activity compared with doxycycline or cefuroxime controls. Residual viable B. burgdorferi was calculated according to the regression equation and ratios of Green/Red fluorescence obtained by SYBR Green I/PI assay.*

[c] *One of triplicate subculture samples grew up, and the other two samples did not grow back.*

[d] *Samples were sterile through 0.22 μm filter.*

EE, ethanol extract; WE, water extract.

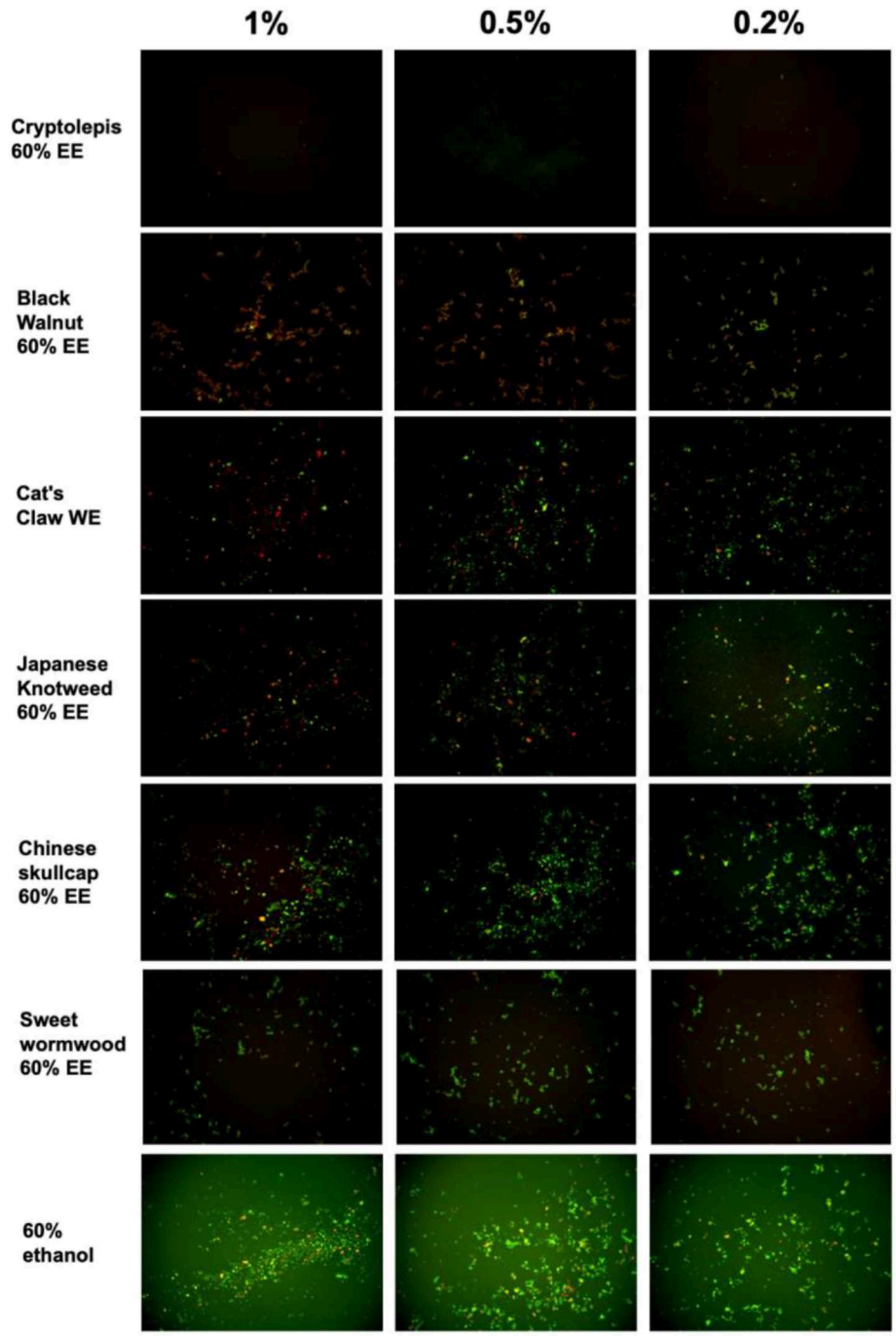

FIGURE 1 | Effect of natural product extracts on the viability of stationary phase *B. burgdorferi.* A 7-day old *B. burgdorferi* stationary phase culture was treated with the natural product extracts at 1, 0.5, and 0.2% for 7 days followed by staining with SYBR Green I/PI viability assay and fluorescence microscopy.

Subculture Studies to Evaluate the Activity of Natural Product Extracts Against Stationary Phase *B. burgdorferi*

To confirm the activity of the natural product extracts in eradicating the stationary phase *B. burgdorferi* cells, we performed subculture studies as previously described (30). We further tested the top active natural product extracts (*Cryptolepis sanguinolenta*, *Polygonum cuspidatum*, *Artemisia annua*, *Juglans nigra*, and *Scutellaria baicalensis*) to ascertain if they could eradicate stationary phase *B. burgdorferi* cells at 1 or 0.5% by subculture after the treatment (**Table 2**). Treatment with 1% *Cryptolepis sanguinolenta* extract caused no regrowth in the subculture study (**Table 2** and **Figure 2**). However, the

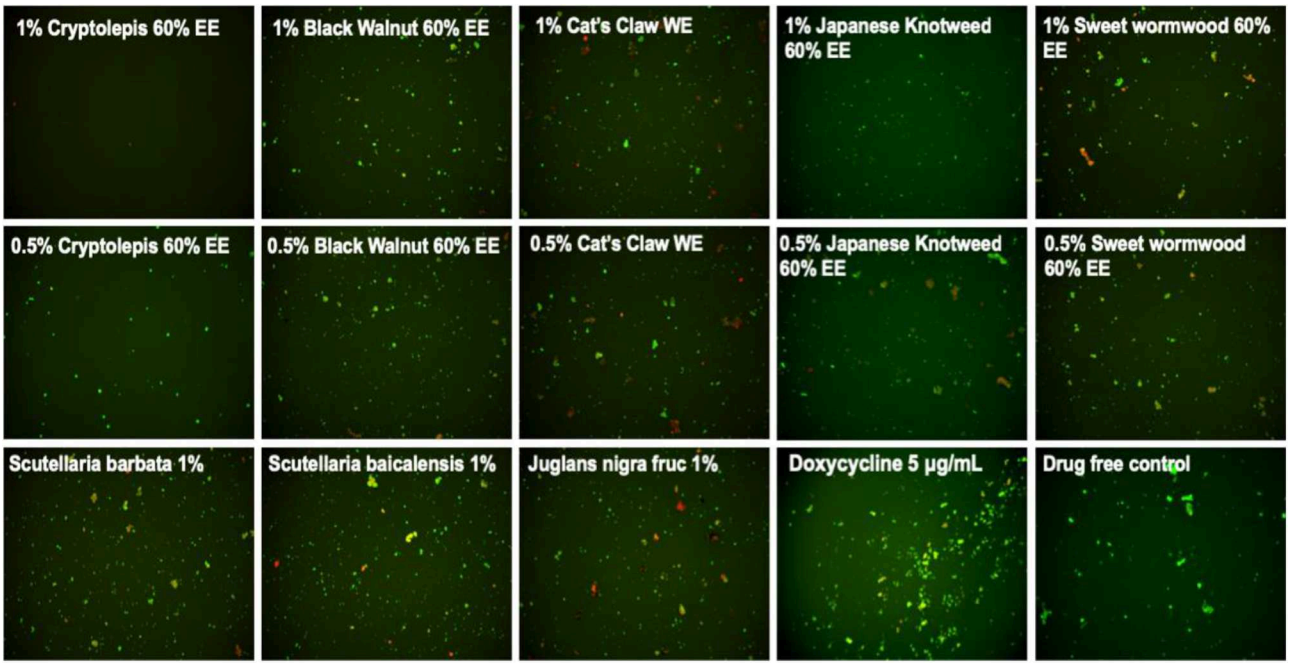

FIGURE 2 | Subculture of *Borrelia burgdorferi* after treatment with natural product extracts. A 7-day stationary phase *B. burgdorferi* culture was treated with the indicated natural product extracts for 7 days followed by washing and resuspension in fresh BSK-H medium and subculture for 21 days. The viability of the subculture was examined by SYBR Green I/PI stain and fluorescence microscopy.

other natural product extracts including *Polygonum cuspidatum*, *Artemisia annua*, *Juglans nigra,* and *Uncaria tomentosa* could not eradicate *B. burgdorferi* stationary phase cells as many spirochetes were still visible after 21-day subculture (**Table 2** and **Figure 2**). At 0.5%, all the natural product extracts treated samples grew back after 21-day subculture (**Table 2** and **Figure 2**), however, only one of the three *Cryptolepis sanguinolenta* extract treated samples grew back. This indicates that 0.5% *Cryptolepis sanguinolenta* extract still has strong activity and could almost eradicate the stationary phase *B. burgdorferi* cells. By contrast, the clinically used antibiotics doxycycline and cefuroxime at clinically relevant concentration (5 µg/ml) could not sterilize the *B. burgdorferi* stationary phase culture, since spirochetes were visible after 21-day subculture (**Table 2**).

DISCUSSION

In this study, we evaluated a panel of botanical medicines and natural products commonly used by some patients to manage their persisting symptoms of Lyme disease and found that indeed some of them have strong activity against *B. burgdorferi.* These include *Cryptolepis sanguinolenta*, *Polygonum cuspidatum*, *Juglans nigra*, *Artemisia annua*, *Uncaria tomentosa*, *Cistus incanus*, and *Scutellaria baicalensis*. The antimicrobial activities of these 7 active herbs are presented in **Supplementary Table 1**. These findings may provide a basis for the clinical improvement of patients who take these medicines and also indirectly suggest their persisting symptoms may be due to persistent bacteria that are not killed by conventional Lyme antibiotic treatment. Surprisingly, *Andrographis paniculata*, *Stevia rebaudiana* (44),Colloidal silver (Argentyn 23^{TM}), Monolaurin (Lauricidin^{TM}), *Dipsacus* spp., and *Withania somnifera,* which are assumed or previously reported to have anti-borrelia activity, did not show significant activity against either stationary phase or growing *B. burgdorferi* in this study.

Cryptolepis sanguinolenta is a plant indigenous to Africa where it has been used in traditional medicine to treat malaria, tuberculosis, hepatitis, and septicemia (45). *Cryptolepis sanguinolenta* has been shown in preclinical studies to have anti-inflammatory (46, 47) antibacterial (48–50), anti-fungal (51), anti-amoebic (52), and anti-malarial (53, 54) properties. Two preliminary clinical studies have documented significant efficacy in treating uncomplicated malaria without signs of overt toxicity (55). While multiple secondary metabolites with antimicrobial activity have been identified, an alkaloid called cryptolepine has been the most well-studied to date. Cryptolepine's antimicrobial activity is thought to be secondary to multiple mechanisms of action including both bactericidal and bacteriostatic effects (48). More specifically, cryptolepine has been shown to cause morphologic changes and cellular breakdown (51), as well as DNA intercalating and topoisomerase II inhibiting effects (56, 57). It should be noted that, in addition to cryptolepine, other constituents in *Cryptolepis sanguinolenta* have also been shown to have antimicrobial activity (58).

Cryptolepis sanguinolenta is generally well-tolerated and few side effects have been documented in humans during its relatively long-term use in parts of China and India. Rat studies indicate that doses of the extract up to 500 mg/kg are relatively safe (59).

Importantly, a novel finding of this current study is the fact that *Cryptolepis sanguinolenta* has strong activity against growing *B. burgdorferi* with low MIC and also non-growing stationary phase *B. burgdorferi* (**Table 2** and **Figures 1**, **2**). Given its traditional use against malaria, in the Lyme treatment community *Cryptolepis sanguinolenta* has been used for treatment of *Babesia* spp. (60) which can be a co-infecting malaria like organism. To our knowledge, the anti-*Borrelial* effect of *Cryptolepis sanguinolenta* has not previously been documented and further *in vitro* and *in vivo* studies are warranted to investigate the potential role *Cryptolepis sanguinolenta* may serve in the treatment of Lyme disease.

Juglans nigra and its constituents have been shown to have antioxidant, antibacterial, antitumor and chemoprotective effects (61, 62). Previous *in vitro* testing has documented that *Juglans nigra* exhibited bacteriostatic activity against log phase spirochetes of *B. burgdorferi* and *B. garinii* and bactericidal activity against *Borrelia* round bodies (63). Two different commercially available botanical formulations which contain *Juglans nigra* were also recently shown to have activity against log phase spirochetes of *B. burgdorferi* strain GCB726, round bodies and biofilm formation in *in vitro* testing (64). *Juglans nigra* has also been shown to have multiple constituents (65) with antimicrobial properties including juglone (5-hydroxy-1,4-naphthalenedione), phenolic acids, flavonoids, and catechins (including epigallocatechin gallate) (66, 67). Further studies are needed to elucidate which constituents have anti-borrelial activity. *Juglans nigra* is well-tolerated and side effects are uncommon. In some individuals, it can cause gastrointestinal disturbance (68) and induce changes in skin pigmentation (69, 70). There can be some allergic cross reactivity in those allergic to tree nuts or walnuts, as well as cases of dermatitis reported in humans (71). The active compound juglone was found to have an oral LD50 in rats of 112 mg/kg (72).

Polygonum cuspidatum has documented anti-tumor, antimicrobial, anti-inflammatory, neuroprotective, and cardioprotective effects (73, 74), with the polyphenol resveratrol being one of the main active constituents. Previous *in vitro* testing has documented that resveratrol exhibited activity against log phase spirochetes of *Borrelia burgdorferi* and *Borrelia garinii,* minimal activity against borrelia round bodies, and no significant activity against borrelia associated biofilms (63). Another active constituent, Emodin (6-methyl-1,3,8-trihydroxyanthraquinone), has documented activity against stationary phase *B. burgdorferi* cells (75). Additionally, preclinical research has documented additional antibacterial and anti-biofilm effects (76, 77). The antibacterial activity of *P. cuspidatum* has been attributed to its stilbenes (including resveratrol) and hydroxyanthraquinone content (78). *Polygonum cuspidatum* has been found to have minimal toxicity in animal and human studies. Gastrointestinal upset and diarrhea can occur but resolves with decreasing or stopping the intake (79). While few studies have been performed in humans, a 2010 review found that it is well-absorbed, and rapidly metabolized.

Artemisia annua (Sweet wormwood also called Chinese wormwood and Qing Hao) is a medicinal plant that has been used for medicinal purposes for over 2,000 years (80) and the isolation of an active constituent called artemisinin was awarded the Nobel Prize in 2015 for its role in treating malaria (81). Artemisinin also has prior documented activity against stationary phase *B. burgdorferi* persisters in *in vitro* models (32, 82). Furthermore, a small pilot study demonstrated that a synthetic analog to artemisinin, called artesunate, showed a significant reduction in short term memory impairment in patients with Lyme disease when combined with intravenous ceftriaxone (83). Artemisinin's antimicrobial mechanism of action is not completely understood (84), but is thought to be related to its ability to generate free radicals that damage proteins (85, 86). The artemisinin content of the *Artemisia annua* sample used in the present study was confirmed to be 0.11% by high-performance liquid chromatography/UV-visual spectroscopy at the Institute for Food Safety and Defense (Centralia, WA). High quality *Artemisia annua* should generally contain >0.3% artemisinin. Despite potential suboptimal levels of artemisinin present in the *Artemisia annua* used for the present study, both 60 and 90% alcohol extracts of *Artemisia annua* exhibited better activity against stationary phase *B. burgdorferi* compared to the control antibiotics cefuroxime and doxycycline. One explanation for these results could be that constituents other than artemisinin are important in providing antimicrobial effects, a finding supported by prior studies (59, 87). *Artemisia annua* is generally considered safe provided that the product administered has minimal or no thujone and other terpene derivatives that are potentially neurotoxic (88). Rat studies found that the NOAEL (no-observed-adverse-effect-level) of *Artemisia annua* extract was estimated to be equivalent to 1.27 g/kg/day in males and 2.06 g/kg/day in females) or more (89). In humans, *Artemisia annua* has been used safely in doses up to 2,250 mg daily for up to 10 weeks (88), and 1,800 mg daily have also been used safely for up to 6 months (88). Some gastrointestinal upset including mild nausea, vomiting (more rare), and abdominal pain can occur at higher doses (60). The use of whole plant extracts instead of single constituents offers potential advantages including providing multiple mechanisms of action and synergistic effects that can reduce the risk of developing microbial resistance. An emerging example of this can be seen in malaria treatment where significant resistance has been reported with artemisinin-based combination therapy (ACT) (90, 91), whereas preliminary studies show improved efficacy and reduced side-effects when treatment with the whole *Artemisia* plant is used (87, 92).

Scutellaria baicalensis and its constituents have been shown to have neuroprotective, antioxidant, anti-apoptotic, anti-inflammatory, and anti-excitotoxicity activity (93–96). One of the active constituents found in *Scutellaria baicalensis*, baicalein, was found to exhibit *in vitro* activity against various morphologic forms of *B. burgdorferi* and *B. garinii*, including log phase spirochetes, latent round bodies, and biofilm formations (97). Additional research has further documented antimicrobial activity (98), synergistic effects with antibiotics (99–101), and reduced biofilm formation (102). *Scutellaria baicalensis* has documented clinical safety (103, 104). There are reports of sedation and it has been shown to be active on the GABA receptor sites (105, 106). A medical food combination of purified *Scutellaria baicalensis* and the bark of *Acacia catechu* containing concentrated baicalin and catechin (Limbrel™, Move Free Advanced™) caused reversible liver damage in at least 35

cases, with a calculated estimated incidence of approximately 1 in 10,000 (107). Despite the case reports of hepatotoxicity, a dose of 1,000 mg/kg daily was identified as the no-observed-adverse-effect level (NOAEL) for this commercial product (108). Hepatotoxicity is generally not seen from the whole plant extract and in a recent study no hepatotoxicity was found in patients taking 1,335 mg per day for an average of 444 days (109).

Uncaria tomentosa has documented neuroprotective effects in preclinical studies (110), and preliminary human studies have shown improved quality of life in individuals with cancer (111), enhanced DNA repair (112), and symptom improvement in individuals with rheumatoid arthritis (113) and osteoarthritis (114). The potential antimicrobial effects of *Uncaria tomentosa* have not been widely evaluated. In a non-peer reviewed publication, *Uncaria tomentosa* was reported to have anti-borrelial effects in an *in vitro* model (115). *Uncaria tomentosa* has also been shown in peer reviewed research to have antimicrobial effects against human oral pathogens (116). *Uncaria tomentosa* has been found to be safe and to have minimal side effects in a variety of animal and human studies (112). Human studies ranging from 4 weeks (114) to 52 weeks (113) demonstrated side effects comparable to placebo. While gastrointestinal complaints such as nausea, diarrhea, abdominal pain, and anemia, were reported, it was thought that study patients had experienced health issues from their solid tumor disease progression and not necessarily from the *Uncaria* (111). The acute median lethal dose in mice was >16 g/kg body weight (117).

It has been proposed that *Cistus incanus* and *Cistus creticus* are synonymous (www.theplantlist.org) while other sources have suggested that *Cistus creticus* is a subspecies of *Cistus incanus* (118). Preliminary clinical studies have shown significant improvement in upper respiratory infection and inflammatory markers in patients taking *Cistus incanus* (119), a volatile oil extract of *Cistus creticus* has been shown to have anti-borrelial effects in an *in vitro* model (120). Additional *in vitro* studies have documented the antimicrobial effects of *Cistus creticus* against several bacteria (118, 121). *Cistus creticus* also demonstrated significant inhibition of *Streptococcus mutans* biofilm formation (121) and reduction in bacterial adherence to enamel (122). *Cistus creticus* has been shown to contain several active constituents (123), including carvacrol (120). Given that our lab previously documented carvacrol to have a significant activity against log and stationary phase *B. burgdorferi* cells (41), it is possible that the carvacrol content in the *Cistus incanus* sample tested in the present study contributed to the significant reduction in log and stationary phase *B. burgdorferi* cells in the present study. *Cistus incanus* plant extracts have been used for centuries in traditional medicine without reports of side effects or allergic reactions (124). In a randomized placebo-controlled study of 160 patients, 220 mg per day *Cistus incanus* was well-tolerated with less adverse effects than in the placebo group (119). While pharmacokinetic safety data is sparse, a cell culture study showed that *Cistus incanus* did not cause any adverse changes on cell proliferation, survival, or cellular receptor function (124).

Grapefruit seed extract (GSE) was previously reported to have *in vitro* activity against motile and cystic morphologic forms of borrelia bacteria in an *in vitro* model (125). In contrast, the current study did not demonstrate meaningful activity against *B. burgdorferi*. There are several potential reasons to explain the difference in results between the current study and previous study including differences in GSE formulations and/or different borrelia species used in culture. In the current study we used *B. burgdorferi* strain B31 whereas the 2007 study states that "*B. afzelii* ACA-1" was used. While both studies used Citrosept™ brand GSE, the formulation has been modified and currently holds an "organic" designation. Because previous studies have documented several contaminants in commercial GSE formulations, including Benzalkonium chloride, triclosan, and methylparaben (126, 127), we screened the GSE products for contaminants prior to inclusion in our present study. The Citrosept™ sample was found to have no detectable levels of contaminants and therefore was used as the GSE source in the current study. In contrast, a second commercially available brand of GSE (Nutribiotic™) did test positive for elevated levels of Benzalkonium chloride, which is a known antimicrobial compound (128) and has been implicated in drug-herb interactions causing potential safety concerns for patients taking GSE (129). The 2007 study did not note testing for contaminants, so it is possible that the previous formulation of Citrosept™ contained a contaminant that exerted anti-borrelial activity.

Stevia rebaudiana was recently reported to have strong anti-borrelia activity (44). However, in our testing, *Stevia rebaudiana* failed to show any activity against *B. burgdorferi.* One possibility to explain this discrepancy is that the study that reported *Stevia rebaudiana* having activity against *B. burgdorferi* did not have appropriate alcohol control. Hypothetically, the previously documented anti-borrelial effect seen may have been due to a non-specific alcohol effect on the *Borrelia* bacteria and not due to *Stevia rebaudiana* itself, or due to differences in plant species, growing conditions, or how the herb is processed. Since we obtained our *Stevia rebaudiana* preparation from an experienced herbalist who extracted it using a known concentration of alcohol, we worked with a preparation with known alcohol concentration. When we used proper alcohol controls we did not find *Stevia rebaudiana* to have any activity against *B. burgdorferi* (**Table 2**).

Andrographis paniculata has been used to treat the spirochetal infection leptospirosis (36) and is anecdotally used by patients with Lyme Disease (60). However, we found Andrographis failed to show any activity against *B. burgdorferi* in our testing. It is possible that *Andrographis* indirectly acts on the host immune system to kill *B. burgdorferi* or induces a non-specific host response. Further studies are needed to test the possible effect of *Andrographis* on the host immune cells.

While this current study has identified novel new botanical and natural medicines with *in vitro* anti-*Borrelia* activity, it is also notable that many herbs or compounds tested did not show direct anti-*Borrelia* activity despite the fact that they are widely used, with anecdotal reports of clinical effectiveness, by patients and practitioners in the community setting (https://www.lymedisease.org/mylymedata-alternative-lyme-disease-treatment/) (60). It is important to consider the potential limitations of the *in vitro* model given that it exists outside of the biological organism. The *in vitro* model can provide

information on direct antimicrobial activity, and while this can be part of the function of botanical and natural medicines, they can also function via additional diverse pathways. For example, they can exert effects via anti-inflammatory/anti-cytokine activity, immune system regulation/augmentation, adaptogenic stimulation of cellular, and organismal defense systems, and biofilm disruption to name a few. In these activities, the mechanisms of the medicines rely on complex interplay and interaction between different body systems, which can only occur within the living organism. Because the *in vitro* model is unable to provide information with regards to alternative pathways through which natural botanical medicines act, it is important that future *in vivo* studies be performed to investigate the activity and efficacy of these and other botanical and natural medicines against Borrelia and other tick-borne diseases. These types of studies will be of vital importance given the multiple factors at play with the current epidemic of tick-borne diseases in our society and globally. While research is beginning to provide information on novel antibiotic combinations that might be effective against the multiple forms of the *Borrelia* bacteria (31), there is ongoing concern regarding extended antibiotic use and care is required regarding issues of responsible stewardship of antibiotic use and antibiotic resistance. It is also important to recognize that, while being cognizant of specific side effects and interactions, botanical and natural medicines generally have a favorable safety profile compared to prescription antibiotics and have a broader spectrum of action with multiple synergistic compounds present within a single plant. Furthermore, using multiple botanical medicines in combination can further increase synergy and efficacy and lower the risk of pathogen resistance development.

CONCLUSION

In conclusion, we tested a panel of botanical and natural products that are commonly used by Lyme disease patients and found several to be highly active *in vitro* against stationary phase *B. burgdorferi* including *Cryptolepsis sanguinolenta*, *Juglans nigra*, *Polygonum cuspidatum*, *Uncaria tomentosa*, *Artemisia annua*, *Cistus creticus*, and *Scutellaria baicalensis*. In contrast, we found that *Stevia rebaudiana*, *Andrographis paniculata*, Grapefruit seed extract, colloidal silver, monolaurin, and antimicrobial peptide LL37 had little or no activity against *B. burgdorferi* in our *in vitro* model.

Since traditional antibiotic approaches fail to resolve all symptoms in a subset of patients treated for Lyme disease, there is a need for developing novel treatment strategies including identifying antimicrobial agents that are effective against persister microcolonies of *B. burgdorferi*. Future studies are needed to further evaluate the seven active botanical medicines identified in the present study as having better activity than doxycycline and cefuroxime against stationary phase *B. burgdorferi*. Specifically, studies should be directed at identifying the active constituents of each botanical, evaluating synergistic combinations, and confirming safety and efficacy in animal models and subsequent clinical studies.

DATA AVAILABILITY STATEMENT

All data sets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YZ, JF, JL, and SS conceived the experiments, analyzed the data, and wrote the paper. JF performed the experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00006/full#supplementary-material

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Conflict of Interest: JL is owner of two naturopathic medical practices, FOCUS Health Group and Door One Concierge, which provides treatment to patients with tick-borne diseases. JL does receive profits from medical services and botanical preparations he exclusively makes available to patients in these two practices and does not currently sell botanical products commercially.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Medicine

REVIEW
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Post-treatment Lyme Disease as a Model for Persistent Symptoms in Lyme Disease

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It has long been observed in clinical practice that a subset of patients with Lyme disease report a constellation of symptoms such as fatigue, cognitive difficulties, and musculoskeletal pain, which may last for a significant period of time. These symptoms, which can range from mild to severe, have been reported throughout the literature in both prospective and population-based studies in Lyme disease endemic regions. The etiology of these symptoms is unknown, however several illness-causing mechanisms have been hypothesized, including microbial persistence, host immune dysregulation through inflammatory or secondary autoimmune pathways, or altered neural networks, as in central sensitization. Evaluation and characterization of persistent symptoms in Lyme disease is complicated by potential independent, repeat exposures to *B. burgdorferi*, as well as the potential for co-morbid diseases with overlapping symptom profiles. Antibody testing for *B. burgdorferi* is an insensitive measure after treatment, and no other FDA-approved tests currently exist. As such, diagnosis presents a complex challenge for physicians, while the lived experience for patients is one marked by uncertainty and often illness invalidation. Currently, there are no FDA-approved pharmaceutical therapies, and the safety and efficacy of off-label and/or complementary therapies have not been well studied and are not agreed-upon within the medical community. Post-treatment Lyme disease represents a narrow, defined, mechanistically-neutral subset of this larger, more heterogeneous group of patients, and is a useful definition in research settings as an initial subgroup of study. The aim of this paper is to review the current literature on the diagnosis, etiology, risk factors, and treatment of patients with persistent symptoms in the context of Lyme disease. The meaning and relevance of existing patient subgroups will be discussed, as will future research priorities, including the need to develop illness biomarkers, elucidate the biologic mechanisms of disease, and drive improvements in therapeutic options.

Keywords: Lyme disease, post-treatment Lyme disease, review, clinical presentation, research priorities

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BACKGROUND

Lyme disease is a geographically expanding, vector-borne disease which is transmitted to humans through the bite of a tick infected with various genospecies of the spirochete bacteria *B. burgdorferi* sensu lato (1, 2). The species of *Ixodes* ticks which transmit the disease are commonly found throughout temperate regions of North America, Europe, and Asia (2). Currently, the Centers

for Disease Control and Prevention (CDC) estimate approximately 300,000 new cases of Lyme disease in the United States alone each year (3). However, due to climate change, shifting land use patterns, and the relative abundance and distribution of reservoir hosts, it is anticipated that the geographic range of the tick vector will continue to expand (4, 5). For instance, the number of reported cases in Canada has increased six-fold over the past decade, with particular increases in the eastern provinces of Nova Scotia and Ontario (6, 7).

Clinically, Lyme disease presents with dermatologic and/or viral-like signs and symptoms such as intermittent fever, sweats, chills, malaise, fatigue, and achiness during the acute phase, which can transition to neurologic, cardiac, and/or joint involvement in later stages of the infection as the bacteria disseminate hematogenously (8). Along with these objective signs, persistent and recurrent symptoms such as fatigue, sleep disruption, arthralgia, myalgia, and headache are also commonly present during later stages of untreated Lyme disease and may account for the majority of the patient symptom experience (9). For example, patients with intermittent bouts of late Lyme arthritis continued to have such symptoms present during the intervening intervals (10). Occasionally, symptoms without physical exam, laboratory, or other so-called "objective" findings remain the major or only manifestations of untreated Lyme disease infection (11). The use of direct tests such as culture, polymerase chain reaction (PCR) or antigen detection for *B. burgdorferi* to aid clinicians in diagnosis is extremely limited, and *B. burgdorferi* cannot be cultured in non-research settings. A two-tier antibody test is widely available and utilized despite significant sensitivity limitations, particularly in early infection and in the convalescent phase after antibiotic treatment of early Lyme disease (12, 13). All stages of Lyme disease are currently treated with antibiotics (14).

The majority of patients return to their pre-morbid health following recommended antibiotic treatment for Lyme disease. However, it has long been observed in clinical practice and in research settings that a subset of patients continue to report a constellation of largely patient reported, so-called "subjective" symptoms which may last for a significant period of time following treatment (15–24). Nevertheless, the epidemiology, significance, etiology, and appropriate treatment of these persistent symptoms are not well-understood and as such, remain the subject of a great deal of scientific dispute and controversy within the medical community (25–28). Patients who can be said to have post-treatment Lyme disease (PTLD) (also called post-treatment Lyme disease syndrome or post-Lyme disease syndrome) represent a narrow, highly specific subset of the broader population of patients with persistent symptoms (14). This specificity is important in research, but not always in clinical settings, as there are multiple pathways through which patients who may be suffering from on-going symptoms from Lyme disease may not meet these narrow criteria. The term PTLD is neutral to underlying disease mechanism and as such, we do not necessarily assume that patients with PTLD have achieved microbiologic cure with initial antibiotic therapy. The aim of this manuscript is to review the published literature from a variety of academic disciplines and perspectives on symptoms which persist or recur in the setting of Lyme disease. We acknowledge that this is a broad topic, and one limitation of our manuscript is that not all related concepts could be readily addressed due to space constraints.

ESTIMATED FREQUENCY

After Lyme disease was first identified in the United States in the late 1970's, but before the pathogenic bacteria was recognized, it was noted that untreated patients with Lyme arthritis often also reported concurrent symptoms such as headache, fatigue, myalgia, and hyperesthesia (9). It was first reported in some of the earliest cases series of treated patients that these symptoms could persist following antibiotic therapy (29, 30). Among patients diagnosed and treated in the early to mid-1980's, largely with penicillin and/or tetracycline, up to 50% experienced symptoms such as fatigue, musculoskeletal pain, memory impairment, and headache several years after treatment (29, 31, 32). A large, population-based study on Nantucket Island found that 36% of those with Lyme disease contracted and treated in the late 1980's had on-going symptoms six years later, and that they were significantly more likely than those without a history of Lyme disease to report fatigue, headache, cognitive complaints, sleep disturbance, and musculoskeletal pain, numbness and/or weakness (33).

As more effective antibiotic treatments and drug regimens were tested and identified in prospective studies and clinical treatment trials, other investigators reported estimates of 0 to 35% for persistent, non-specific symptoms following treatment (15–24). These symptoms were often considered "minor" and classified independently from defined treatment failure; objective signs of neurologic, cardiac, or joint involvement which would indicate progression to later stages of the infection. This relatively broad range of estimates is likely a reflection of several of the study design challenges which are still relevant in the field today. First, inter-study variability in enrollment criteria may encompass factors directly related to risk of persistent symptoms (see section Risk Factors). For instance, studies which require an active erythema migrans (EM) rash at enrollment will by definition exclude patients with longer disease durations, a likely risk factor for persistent symptoms. Population-based studies may be more reflective of the community practice of medicine than those conducted in academic research centers, with a wider range of treatment regimens, a higher misdiagnosis rate, and longer duration of disease prior to appropriate antibiotic treatment. Finally, without an objective biomarker, there has been a lack of standardization in outcome ascertainment, with many studies relying on physician assessment and classification into subjective sub-categories.

While Lyme disease has been a nationally notifiable disease in the United States since 1991, the CDC does not track disease outcomes or cases of persistent symptoms (34). Estimating the population-level prevalence of persistent symptoms following Lyme disease is challenging due to this lack of standardization or consensus in operationalizing a case definition. Furthermore, it is hindered by the difficulty of obtaining valid incidence rate

estimates of new Lyme disease infections, as Lyme disease has traditionally been tracked through passive surveillance which has historically led to significant under-reporting of cases (35). One recent study attempted to estimate cumulative prevalence of persistent symptoms after treatment using statistical simulation techniques (36). The authors estimate almost 1 million cases by 2020, assuming continued linear growth of new Lyme disease cases since 1980 and a potentially conservative 10% "failure" rate of new infections.

RISK FACTORS

Several clinical factors surrounding the initial onset of Lyme disease have been found to increase risk for persistent symptoms after treatment. More severe disease at onset in the form of a higher number of symptoms (37) and/or objective signs (such as Bell's Palsy) or symptoms (such as headache, photophobia, or neck pain) which suggest dissemination to the nervous system may be present and may increase the risk of persistent symptoms following treatment (33, 38). One recent study has shown that the presence of pre-existing co-morbidities in Lyme disease was predictive of long-term symptoms and lower quality of life (39), similar to other disease settings (40, 41). Delays in diagnosis and initiation of appropriate treatment, which importantly may be driven by patient health insurance status (42), have also been shown to increase risk (31, 32). Diagnostic delays may also be compounded in some initially misdiagnosed patients by subsequent exposure to inappropriate or ineffective treatments (15, 43). While it is unknown whether corticosteroid exposure during acute infection, often prescribed for associated facial palsies, may affect resolution of systemic symptoms, it has been shown to be associated with worse long-term facial function outcomes (44, 45). Although awareness of Lyme disease has increased in recent decades, the wide range of clinical heterogeneity at presentation and the limited sensitivity of the two-tier test mean that misdiagnosis and delays in diagnosis still occur with some frequency in the community practice of medicine (42, 43).

Several studies have also suggested that factors relating to the initial immune response to infection may drive later clinical outcomes after treatment. A muted immune response during acute infection, in the form of lower levels of circulating plasmablasts, has been associated with persistent symptoms after treatment (46). However, elevated levels of specific immune mediators such as IL-23 and CCL19 at disease onset and/or in the immediate convalescent period have been associated with the presence of persistent symptoms up to 1 year following treatment (47, 48). It is unclear whether the magnitude of the initial antibody response to *B. burgdorferi* prior to treatment is of importance, as a negative serology has been found to be both associated and not associated with subsequent clinical outcomes (15, 37).

Additionally, while the detailed biology of *B. burgdorferi* is a complex topic (49) which is outside the scope of this article, it has also been hypothesized that specific microbiologic factors may influence treatment outcomes, as over 50 distinct genotypes of *B. burgdorferi* senso stricto have been identified across North America and Europe (50). While associations between infecting genotype and early disseminated disease have been identified (51, 52), more research is needed on potential associations with treatment outcomes. One small study of 14 patients did not find a pattern in the specific genotypes of the infecting *B. burgdorferi* strain among patients with persistent symptoms a decade after treatment for Lyme disease (24). However, RST1 strains which have been found to be more highly inflammatory are associated with more severe symptoms and increased risk of antibiotic-refractory arthritis (53, 54). Furthermore, *Ixodes* ticks are capable of simultaneously carrying and transmitting multiple genotypes of *B. burgdorferi* (55, 56), as well as multiple distinct pathogens. The effects of both of these on risk of persistent symptoms after treatment have not been studied comprehensively, however they may worsen treatment outcomes. In one study, patients co-infected with *B. burgdorferi* and *Babesia microti* experienced not only a greater number and diversity of symptoms, but also took longer to resolve both the signs and symptoms of their illness as well as to be clear of spirochete-specific DNA on PCR testing (57).

Among CDC-reported cases of new Lyme disease infections, there is a slight majority male and a bimodal age distribution among younger children and older adults (58). Similarly, there does not appear to be a significant difference by gender among those who meet a highly-specific definition for persistent symptoms in the research setting (59, 60). However, it has been noted that when less specific definitions are used, as may be applied in clinical practice, the ratio of patients is instead majority female (11, 61). When diagnosed and treated promptly, children appear less likely than adults to report persistent symptoms following Lyme disease (22, 62–64) Among adults, it is not clear if age represents a significant risk factor. Among a sample of culture-confirmed patients with EM, those 50 and older at onset of Lyme disease were not significantly more likely than those under 50 to later report persistent symptoms (65). However, in a recent insurance claims analysis of a large, integrated health system in Pennsylvania, members who met a definition for persistent symptoms following an incident Lyme disease diagnosis were more likely to be older (and female) than those who did not (66).

CLINICAL PRESENTATIONS

Currently, there are no commonly agreed-upon symptoms, laboratory, or imaging findings which are sensitive and specific to aid in the clinical evaluation of patients with persistent symptoms in Lyme disease. Therefore, the clinical diagnosis is primarily one of exclusion, and the current illness must be distinguished both from other systemic inflammatory, rheumatic, malignant and infectious conditions, as well as the effects of co-morbid or pre-existing conditions (39). This raises the possibility of anchoring bias, or the misattribution of either symptoms or positive serologies in low-endemic areas to prior Lyme disease, when in fact the symptoms are caused by a new, unrelated illness (67, 68). While anchoring bias may theoretically play a role in evaluation of patients with persistent symptoms, the extent

of its potential contribution is unknown. Furthermore, care should be taken to differentiate prolonged, persistent symptoms from a new, distinct exposure to *B. burgdorferi*, which is often accompanied by a new EM (69). Finally, as the presence of symptoms alone cannot currently definitively establish the link to prior Lyme disease, a second key component is grounding the current illness to the initial exposure to *B. burgdorferi*. This requires a careful, clinical history for clues in the past medical history that may have been missed, such as a misdiagnosed skin lesion or a non-specific, acute, summer, flu-like illness at the onset of the patient's change in health (70, 71). Above all, a thorough clinical history must also account for inter-personal variability, the potential implications of initial misdiagnosis, and the diagnostic limitations of two-tier testing.

Symptoms

The prolonged, subjective symptoms frequently reported in the context of Lyme disease (e.g., fatigue, widespread pain, cognitive complaints, paresthesia, and sleep disruption) also broadly represent those commonly reported in outpatient settings (72–74). Furthermore, while some differences in impairment and symptom distribution have been studied and reported (75–77), there is also a degree of general symptom overlap with other disease states such as traumatic brain injury, depression, chronic fatigue syndrome, and fibromyalgia (78). This lack of sensitivity can lead to the conclusion that the prolonged symptoms reported in Lyme disease are no different than the "background noise" of symptoms in the general population. However, the magnitude of the symptoms, as well as the number of co-occurring symptoms reported, is often more severe. In our study of participants with well-characterized PTLD compared to a control group with similar age and gender characteristics, 25 of 36 symptoms assessed were found to be statistically significantly more severe in participants with PTLD (**Figure 1**) (60). Health-related quality of life, as measured by the 36-item short-form health survey (79), is typically not only lower than controls, but comparable to other major chronic diseases, such as congestive heart failure (60, 80, 81).

Prolonged, persistent symptoms in Lyme disease are primarily patient reported and are therefore considered subjective. Although objective signs may be present, they are not distributed consistently enough across patients nor are they specific enough to be considered diagnostic. Fatigue is often the most commonly reported, severe symptom with levels comparable to patients with multiple sclerosis on the Fatigue Severity Scale instrument (60, 82, 83). In one study, fatigue was also found to be the most important contributor to levels of physical functioning (81). This suggests that fatigue may be an important primary intervention target for patients, including evaluation of related factors such as sleep quality and mood disturbance. Additionally, a history of orthostatic intolerance may be indicative of postural orthostatic tachycardia syndrome or other autonomic dysfunction, which may be another treatable cause of fatigue (84).

Persistent chronic pain in Lyme disease has been described in various studies as neuropathic or nociceptive (85). It has been noted in the literature that pain among patients with PTLD is uniquely asymmetrical, is more likely to involve the limbs, and is less widespread than in fibromyalgia (86, 87), however to our knowledge these observations have not been systematically examined. Pain and/or stiffness in the neck appears to be a common specific presenting location (60, 88). While also meeting criteria for later fibromyalgia may be rare among patients treated promptly for early Lyme disease as a whole (1%) (87), earlier studies suggested that this overlap may be more common among the subset of patients with prolonged, persistent symptoms (89–91). Neurocognitive complaints, including both behavioral and memory-related issues, are also among the most frequently reported symptoms (33, 60, 92).

Although fatigue, musculoskeletal pain, and cognitive dysfunction are the most commonly reported symptoms, a host of others, including sleep disruption, paresthesia, headache, dizziness, and mood changes, are variably distributed across patients (60, 88, 93). As a whole, patients report that symptoms may wax and wane, or may persist with stable levels of severity. While symptoms in some patients may resolve in the initial convalescent period following treatment, for others they may last for decades after initial exposure (24, 38).

Physical Examination

Among patients with prolonged, persistent symptoms in Lyme disease, the physical examination is often largely normal, and an important initial focus should be to exclude the presence of findings which would suggest another potential cause of the patients' symptoms. Special attention should be paid to the musculoskeletal examination for objective evidence of joint inflammation and swelling. Patients with persistent symptoms after treatment of early localized or early disseminated Lyme disease often have joint pain (i.e., arthralgia) but almost never have inflammatory arthritis with joint inflammation and swelling (33). Oligoarthritis with obvious swelling, especially of the knee, may suggest a site of ongoing infection. Arthrocentesis may then be performed, and the synovial fluid tested by PCR, to evaluate for *B. burgdorferi* infection and the need for further antibiotic therapy. Patients with previously treated late Lyme arthritis however, may have persistent swelling as a manifestation of antibiotic refractory late Lyme arthritis (now also called post-infectious Lyme arthritis), a persistent form of synovitis following antibiotic treatment (53, 94–96). Patients with persistent symptoms who develop polyarthritis with an abnormal joint exam suggestive of rheumatoid arthritis or psoriatic arthritis following treatment for acute Lyme disease have also been described (97). In this case, it is hypothesized that Lyme disease triggered the polyarthritis in susceptible individuals, such as those with a history of psoriasis, but that it is not due to active infection of the joint. Therefore, the aim of treatment should be to control the potentially joint-damaging inflammation using established therapies for managing rheumatoid arthritis or psoriatic arthritis (97).

After treatment for acute neurologic disease such as seventh nerve palsy or radiculititis, the neurologic examination may identify residual deficits, such as cranial nerve damage or radicular pain. These findings may resolve gradually and/or leave residual fixed deficits which may be more or less apparent to the patient from that point on (98). Neurologic findings among

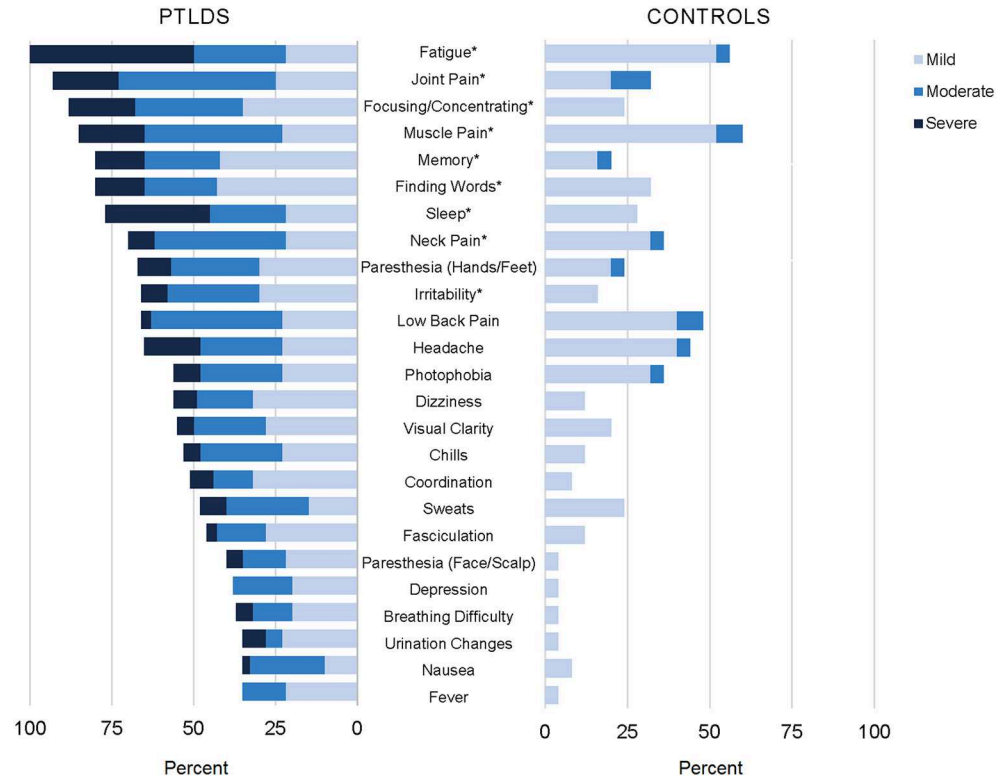

FIGURE 1 | Participants with post-treatment Lyme disease syndrome (PTLDS) and controls were asked about presence and severity of 36 signs/symptoms over the past 2 weeks. Displayed are the 25 signs/symptoms with a statistically significant difference in severity by group ($p < 0.05$), ordered by frequency within the PTLDS group. The nine signs/symptoms with a statistically significant difference at the $p < 0.001$ level are indicated with an asterisk. This figure was originally published and is reprinted from (60) under the Creative Commons CC-BY license.

patients with persistent symptoms are different however, as they are often more subtle and relate more to symptoms and signs of encephalopathy, and may require consultation for uncertain cases. In patients with untreated late Lyme encephalopathy or persistent symptoms with neurologic manifestations, the most common physical findings are related to memory loss or difficulty finding words, which may be documented on neurocognitive testing (see section Neurocognitive Testing). Hyperreflexia and evidence of upper motor neuron weakness is rarely found (99).

After treatment of late Lyme encephalopathy, more than half of patients will improve. In one study 22% improved but then relapsed with what would now be considered PTLD, with symptoms and signs of encephalopathy (99). Antibiotic therapy is typically effective in resolving obvious signs of vertigo, dizziness, and hearing loss found in neuroborreliosis, and persistent balance instability responds well to vestibular rehabilitation (100). Persistent audiologic complaints were found in a small study of 18 patients with PTLD, and 44% had one or more abnormal pure tone threshold, 31% had abnormally reduced loudness discomfort level, and 17% had abnormal acoustic reflexes at one or more frequencies (101).

In untreated patients with persistent sensory or motor symptoms, examination of the peripheral nerves may demonstrate evidence of peripheral neuropathy, which should be confirmed on electromyography or nerve conduction studies (102). In our clinical case series of patients with PTLD, the most common neurologic exam finding was abnormal vibratory sensation. Thirty-two percent were found to be below age-adjusted threshold values for vibratory sense on either upper or lower extremities using a Rydel-Seiffer 64 Hz tuning fork compared to an estimated, expected 5% in the general population (60, 103). Furthermore, although numbness, tingling, paresthesia, and altered temperature perception are common persistent symptoms and often occur in the context of an otherwise normal physical examination, they should prompt consideration of a possible small fiber neuropathy. This may be pursued through a specialized skin biopsy to measure small nerve fiber density, which may also show evidence of autonomic nervous system involvement as well (104). Development of overt postural orthostatic tachycardia syndrome in PTLD is rare but has been described in the literature (84, 105). Tilt table testing for evidence of orthostatic intolerance syndromes may be useful in order to guide specific interventions.

Laboratory and Imaging

Patients with persistent symptoms in the context of Lyme disease should undergo blood work at their initial evaluation, which may include a complete blood count, metabolic panel, thyroid testing,

erythrocyte sedimentation rate, and C-reactive protein. This is important in order to rule out other symptom causes such as severe anemia, liver, kidney or other metabolic conditions such as diabetes, or other inflammatory or neoplastic conditions. Mild elevations in C-reactive protein have been reported in PTLD (106), however moderate to severe elevations in the erythrocyte sedimentation rate or C-reactive protein are distinctly unusual and should prompt evaluation of another infectious, neoplastic, or autoimmune condition, such as polymyalgia rheumatica.

Two-tier testing was developed for surveillance purposes and should not be used alone outside of clinical judgment in diagnosing and treating Lyme disease (107). Similarly, it is neither sensitive nor specific in clinically evaluating persistent symptoms in the context of previous antibiotic treatment. A positive serology is not required as part of the proposed research case definition for PTLD (14), as antibody levels have not been found to be associated with specific clinical outcomes following treatment for Lyme disease (38, 108). Particularly for those diagnosed early in infection with localized disease, patients may be seronegative on acute testing and it is known that antibiotic treatment appears to blunt the development of a later serologic response on convalescent testing (13). Conversely, patients may be seropositive for both immunoglobulin M or G antibody responses years or decades later after resolution of their infection (109). This may lead to misattribution of current symptoms to Lyme disease, and other causes must always be considered and excluded even in the context of a positive antibody test. Furthermore, two-tier or C6 antibody testing cannot be used as a test of microbiologic cure, which may be of particular concern when treating later stages of the infection, or when infection involves the central nervous system, where antibiotic penetration may be suboptimal (110).

While patients with persistent symptoms who have not been specifically treated for Lyme disease would be expected to have a positive serologic response, this is not always observed clinically. Several factors may account for this, including unintended prior antibiotic exposure for an alternative co-morbid or misdiagnosed condition. Alternatively, patients may fail to meet the exact cut-off criteria despite evidence of some antibody response to *B. burgdorferi*. In these patients, with the exception of synovial fluid PCR to confirm the diagnosis of late Lyme arthritis, direct diagnostic tests such as bacterial culture or PCR of the blood or cerebrospinal fluid (CSF) are often either insensitive or unavailable in non-research settings (111). Culture of *B. burgdorferi* remains a challenge in both untreated and treated patients, and it is uncertain whether persister organisms can be cultured at all, as evidenced by animal models (112).

Serologic testing for other infectious agents in patients who are *B. burgdorferi* seronegative or who remain ill after initial treatment for Lyme disease may be indicated in certain circumstances. For instance, in patients with suggestive presentations or risk factors for specific animal exposures, testing for Brucellosis, Q fever, or Bartonellosis may be indicated. Scientific knowledge of the frequency and relevance of exposure to multiple co-infectious agents, such as *Anaplasma*, *Ehrlichia*, and *Bartonella* species of bacteria, *Babesia* parasites, or other *Borrelia* species such as *B. miyamotoi*, in persistent symptoms is limited. Symptomatic co-infection of *B. burgdorferi* and *Babesia microti* is well-documented, and may result in chronic illness, especially in patients with an impaired immune system (113). The role of other *Babesia* species, such as *B. duncani*, in persistent symptoms is unknown (114). Infection with *Bartonella* species of bacteria, which can also cause chronic illness, is thought to result primarily from flea bites, although transmission via ticks is an area of emerging knowledge (115). Finally, there has been speculation that non-vector borne infections such as mycoplasma and Epstein-Barr virus may be involved in the perpetuation of chronic symptoms in patients with Lyme disease (116). It should be noted that a positive serologic test for many infections does not equate to on-going infection, as immunological memory can create long-lasting and even life-long antibodies after active infection is resolved.

In patients with untreated late Lyme encephalopathy or persistent symptoms with neurologic manifestations, lumbar puncture may be employed. In these circumstances, an abnormal CSF warrants neurologic consultation and the potential treatment of neuroborreliosis, depending on the clinical circumstances (117). Central nervous system imaging in patients with late Lyme neuroborreliosis and/or neurologic symptoms has not been definitively characterized and more research is warranted. In patients with late Lyme neuroborreliosis, earlier reports of MRI imaging often showed non-specific white matter lesions (118, 119). However, there are currently no imaging findings considered specific for Lyme neuroborreliosis, and significant overlap exists with other neurologic conditions, particularly multiple sclerosis (120). Patients with persistent symptoms, including those with PTLD, have also been studied using various neuroimaging modalities including MRI (121, 122), SPECT (123, 124), and PET (125) scanning techniques. With the exception of one study (121), all identified abnormalities in a subset of individuals within their respective samples. However, imaging studies in Lyme disease have more recently been called into question as a result of advances in imaging technology, knowledge of age-related white matter changes and potential overlap with the general population, and/or increased specificity of diagnostic criteria (126). Newer research techniques, such as those to image central nervous system inflammation using novel PET imaging (127), may aid diagnosis in the future and provide additional insight into the pathophysiology of persistent symptoms.

Neurocognitive Testing

Several studies have characterized the neurocognitive testing profile of patients with PTLD (32, 128–135), who often complain of memory, focus, concentration, and processing speed difficulties (**Figure 1**). We recently reported that among patients with PTLD who gave adequate test engagement on validity testing, 7% were found to have cognitive impairment using stringent measures relative to population norms. However, when compared instead to education-based estimates of their own pre-morbid functioning, 34% of the sample showed decline (128). Patients with a history of treated Lyme disease (32), and in particular the subset who report persistent symptoms (128–130, 133) often have specific, modest deficits in verbal memory as

a group relative to controls. In one study, these objective deficits were found to be present only in the subset of PTLD patients with abnormal CSF findings, suggesting a neurological basis (133). It has also been noted that patients with PTLD can have deficits in mental activation or information processing speed when initiating a cognitive process, independent of sensory, perceptual, or motor deficits (131). Although mood-related symptoms are often present to a greater degree among patients with PTLD compared to controls (**Figure 1**), depression has not been shown to be associated with performance on memory testing in this population (129). Moreover, patients with PTLDS appear to have more pronounced problems on memory-related tasks when compared to patients with major depressive disorder (132).

ETIOLOGY

B. burgdorferi is a zoonosis which has adapted to living in a mammalian host. In its natural reservoir host, the white-footed mouse, it does not appear to cause symptomatic disease. The genome of *B. burgdorferi* does not appear to code for any known toxins, and as such, it does not have the ability to directly damage host tissue (49). Therefore, the symptoms of Lyme disease can be considered mostly due to the host innate and adaptive immune response to infection. For example, early Lyme disease is characterized by high levels of many immune mediators, which may be beneficial in clearing the infection but also can cause symptoms such as fever and malaise (136). In patients with prolonged, persistent symptoms, the host immune response may become dysregulated through inflammatory or secondary autoimmune pathways, or non-specific immune activation. Other systems, such as central neural pathways and networks, may also be disrupted and have a significant impact on symptoms. The primary driver of this initial dysregulation remains unknown, and it may be dependent on or independent of microbial persistence. It is likely that a variety of factors, many of them overlapping and interacting, contribute to this symptom profile (**Figure 2**). All these illness mechanisms occur in the context of the host genetic and environmental background, variability in the infecting organism, and the illness experience of the patient.

In vitro research shows that *B. burgdorferi*, like many bacteria, can form persister organisms either under antibiotic pressure or during stationary phase growth, and that these persister organisms are antibiotic-tolerant and are less likely to be killed with standard antibiotics (137, 138). The fact that symptoms can persist in previously treated patients, including those with PTLD, does not exclude microbial persistence as a hypothesized etiology of the symptoms; moreover, exposure of *B. burgdorferi* to antibiotics is the basis for *in vitro* models of bacterial persistence (138). It remains unknown therefore, whether standard antibiotic therapy may result in partial treatment or generation of persister organisms that may be involved in the ongoing pathophysiology of persistent symptoms. Mouse, dog, and non-human primate model work show evidence of *B. burgdorferi* persistence by tissue histopathology and PCR in antibiotic treated animals (139–142). These persister organisms have been shown to be metabolically active in RNAseq studies and xenodiagnosis with ticks has demonstrated *B. burgdorferi* transmission from antibiotic-treated animals to immunodeficient mice (112). However, they are not easily cultivatable and Koch's postulates have been difficult to prove (140). An alternate explanation for ongoing inflammation is antigen persistence after complete killing of replicating bacteria. In the mouse model, extensive bacterial debris can be visualized after antibiotic treatment despite eradication of cultivatable organisms (143). These antigens may have the ability to specifically activate host immune cells directly or via non-specific bystander activation pathways (144).

Alternatively, antibiotic-refractory late Lyme arthritis, the most studied post-treatment manifestation of Lyme disease, is thought to be autoimmune in nature, as *B. burgdorferi* can no longer be found in the joint or surrounding tissue in patients who have been thoroughly treated (145). Among patients with this form of inflammatory arthritis, high levels of Th17-associated mediators have been found to correlate strongly with autoantibodies to several Lyme disease-specific autoantigens (63). This suggests that immune dysregulation, in the form of a shift toward autoimmune responses, may contribute to on-going synovitis following antibiotic treatment in the joint (146). Investigators have also shown peptidoglycan persistence in synovial fluid of these patients despite the lack of morphologically intact bacteria (147). It is unclear whether this on-going inflammation is sustained by persistent microbial antigens (148), or even the biologic feasibility of chronic antigen persistence in sequestered sites such as the joint.

Investigation into the role of an ongoing immune response in the symptomatology of patients with persistent symptoms is in its earliest stages. A handful of studies have suggested that inflammatory markers such as C-reactive protein, as well as immune mediators such as CCL19 and IL-23, remain elevated for months after completion of antibiotic therapy among patients with persistent symptoms (47, 48, 106). Anti-neural antibody reactivity is higher in those with persistent symptoms, even among those who are seronegative, compared to those who returned to health after treatment for Lyme disease (149). Another study among post-treatment patients with objective memory impairment found a unique proteomic signature in the CSF with specific differentially increased complement cascade proteins (150).

Finally, infection-triggered, post-infectious syndromes have been described for a variety of viruses (151). Given the clinical similarities between many of these syndromes and persistent symptoms in Lyme disease, including fibromyalgia and chronic fatigue syndrome, it has also been hypothesized that analogous underlying mechanisms may contribute to the symptom profile for all of these conditions (152). Specifically, central sensitization is a process of hyperactivation in the central neural pathways, leading to a more intense response to sensory stimuli, which is experienced as hyperalgesia and/or allodynia (152). The presence of depression or anxiety may be related to these altered neural networks and their associated neurotransmitter changes, or it may also evolve under the patient's lived chronic illness experience, which is often marked by uncertainty and newfound significant functional limitations (153). Although the role of

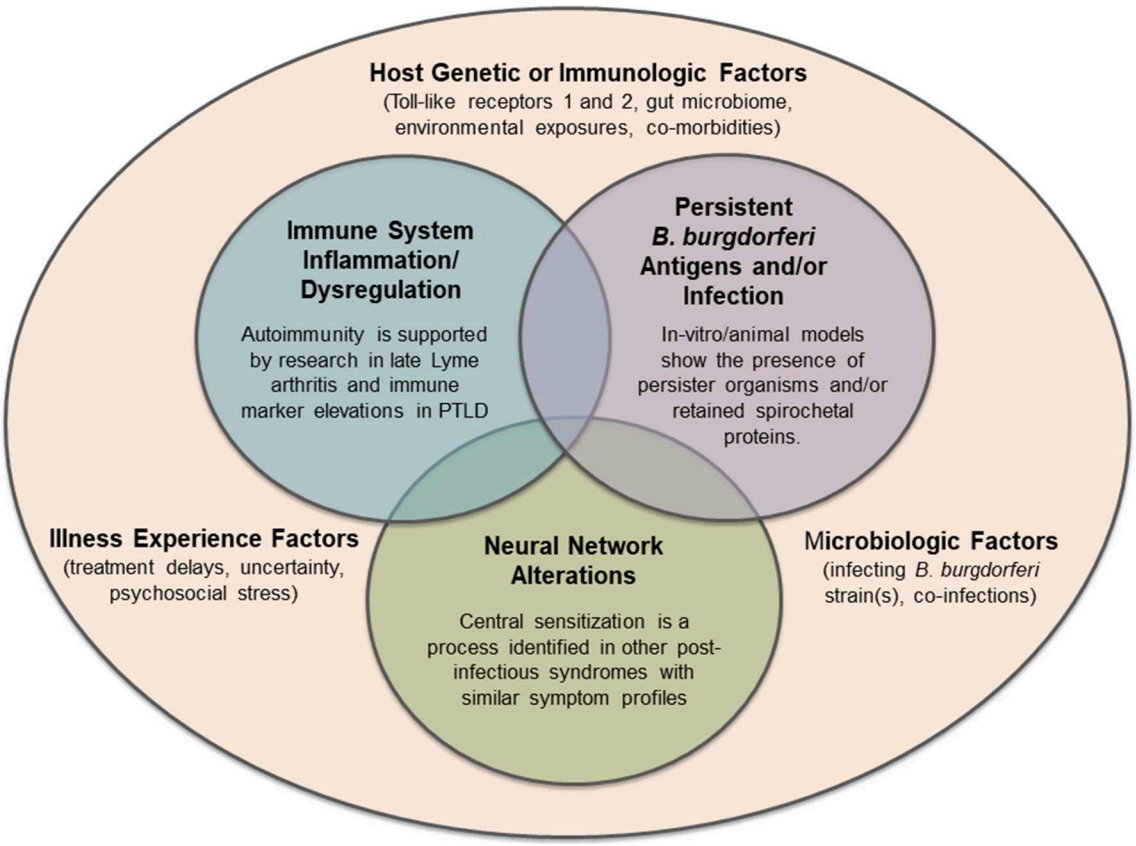

FIGURE 2 | Hypotheses regarding potential mechanisms of persistent symptoms in Lyme disease, all of which may exist within the complex microbiologic, immunologic, genetic, and lived experience of individual patients. Future mechanisms of disease or other pre-disposing factors may still be identified.

central sensitization in fibromyalgia and other syndromes has been previously appreciated, very little research in this area has been conducted among patients with persistent symptoms in Lyme disease.

TREATMENT

There are currently no FDA-approved or commonly agreed-upon treatments for patients who have undergone a recommended course of antibiotics for Lyme disease but who continue to have persistent symptoms. Until the pathophysiology of these persistent symptoms is identified and/or a biomarker is developed, it is likely that treatment recommendations will continue to be without consensus. A small number of double-blind, placebo-controlled clinical trials, with a degree of variability in enrollment criteria, intervention, and outcome measures, have been conducted to test whether additional antibiotics are effective (80, 83, 135, 154, 155). One recent study also tested the added benefit of longer-term compared to shorter term antibiotic re-treatment in PTLD (156). In sum, although the study design and interpretation of clinical relevance in the findings of these studies have been debated (157, 158), they have not provided convincing enough evidence of a significant, sustained treatment effect for the Infectious Diseases Society of America (IDSA) to recommend additional antibiotics in their guidelines (14). Furthermore, anecdotal reports of adverse events or even death (159, 160), and the risk of antibiotic resistance at the population level with long-term, untargeted use of antibiotics are often cited as significant concerns (161). By contrast, the International Lyme and Associated Diseases Society (ILADS) have issued markedly different clinical recommendations focused on often open-ended antibiotic treatment of persistent infection and the potential for multiple tick-borne co-infecting agents (162). These recommendations are based on extensive review of the literature supporting the hypothesis of microbial persistence as a mechanism of persistent symptoms in both untreated and previously treated Lyme disease (163). The debate over appropriate and effective treatment strategies for patients with persistent symptoms is one of the primary drivers of the on-going controversy in Lyme disease.

Aside from antibiotics, additional pharmacologic (albeit off-label) or non-pharmacologic therapies for clinical care focus on managing individual symptoms and restoring or improving functioning. For example, pregabalin, and duloxetine may provide some symptom improvement for patients who also meet criteria for fibromyalgia. Tricyclic antidepressants such as nortriptyline are often used for symptomatic management of

pain and sleep, and selective serotonin reuptake inhibitors may be indicated for management of secondary depression or anxiety. Other medications for fatigue, such as modafinil, may also be considered but none of these interventions have been subjected to controlled trials (164). Non-pharmacologic interventions such as cognitive-behavioral or other types of therapy as a means to ease symptom burden and help manage the stress of living with a chronic illness may be useful as well. Mindfulness-based stress reduction has been tested among patients with fibromyalgia and shows promise in reducing both symptoms and stress levels (165). A supervised resistance exercise program was shown to increase the number of days feeling healthy and energetic among a small sample of patients with persistent symptoms of Lyme disease (166). The use of complementary, alternative therapies such as essential oils may be promising, however additional *in vivo* work is needed to address safety and pharmacokinetic properties (167).

Additional treatment trials are needed to test the effectiveness of new therapeutic approaches for patients with prolonged, persistent symptoms following recommended treatment for Lyme disease. Large drug-screening efforts have identified new potential antibiotic and non-antibiotic therapeutic targets with activity against *B. burgdorferi* (168–170). Although it is unclear how the data will translate to human disease, *in vitro* and animal models support therapeutics which target persister organisms (137, 171). Furthermore, refinement of treatment protocols for antibiotic-refractory late Lyme arthritis have led to a combination of multiple initial, defined courses of antibiotics followed by a transition to anti-inflammatory therapy when the evidence suggests that infection has been eradicated (53). This may provide a model for future testing of anecdotally suggested, defined, retreatment protocols, particularly among patients with risk factors for persistent symptoms, and conceivably with oral antibiotics which have both anti-infective and anti-inflammatory properties (172).

DEFINING PATIENT SUBGROUPS: POST-TREATMENT LYME DISEASE IN THE CONTEXT OF CHRONIC LYME DISEASE

Patients with persistent symptoms related to Lyme disease likely represent a heterogeneous population, which includes previously untreated patients, as well as those treated patients who remain symptomatic. As a result, some (largely those with prior treatment) will manifest primarily patient-reported symptoms while others (largely untreated patients) will present with symptoms in conjunction with objective, physical findings. This heterogeneity is further complicated by variation in terminology and the definitions used by different groups in the field.

Patients with untreated Lyme disease have a significant chance of developing persistent signs and symptoms, primarily in the form of arthritis and less commonly, neurologic disease (2). The best studied and agreed-upon persistent manifestations of untreated Lyme disease are late Lyme arthritis, which may present with joint pain, synovitis, and swelling months to years after initial infection, and its post-treatment sequelae antibiotic-refractory late Lyme arthritis, which may occur in ∼10% of patients (173). These manifestations present with objective joint swelling and the presence of joint fluid which can be analyzed by PCR for *B. burgdorferi*, rendering the diagnosis and biologic evaluation of these conditions possible. Similarly, patients with untreated neurologic disease may develop Lyme encephalopathy, manifesting primarily as memory or other cognitive problems. This symptom complex may require further neurocognitive testing, central nervous system imaging, or CSF analyses for evidence of ongoing infection.

By contrast, the majority of patients with persistent symptoms have primarily patient-reported, non-specific symptoms in the absence of classic physical findings of organ-based damage or disease, and therefore it is often presumed that the source of their illness is unrelated to *B. burgdorferi* exposure (59). In 2007, Feder et al. introduced the concept of multiple sub-categories of patients under the umbrella term "chronic Lyme disease" (CLD) to characterize these patients (174). CLD is a polarizing diagnosis in clinical medicine, with widely divergent definitions and understandings of disease mechanism and effective treatments (175). One key component of the controversy relates to whether CLD is a real disease which is associated pathophysiologically to past or present *B. burgdorferi* infection. We have modified the initial classifications detailed by Feder et al. in the model presented in **Figure 3**, which depicts the subgroups described in this section. These subgroups are primarily distinguished by the strength of the evidence in their past medical history and in their current clinical presentation for exposure to *B. burgdorferi* (174). It is likely that additional, future sub-groups will continue to be identified as our understanding increases of both the pathophysiology of CLD and the diversity of potential infecting tick-borne pathogens.

Patients with PTLD are the most-studied subgroup of those with CLD, as they represent a narrow, highly specific subset of the broader population of patients with persistent symptoms (14, 174). By definition, these patients had documented Lyme disease prior to developing their chronic symptoms which appeared in spite of recommended antibiotic treatment. In 2006, the IDSA included a proposed case definition for PTLD which stipulates the following key elements: (a) a prior documented episode of Lyme disease meeting CDC criteria in which all objective signs resolve; (b) fatigue, widespread musculoskeletal pain, and/or cognitive difficulties which begin within 6 months, last for at least 6 months, and are significant enough to impair daily function; and (c) the absence of specific co-morbid or pre-existing conditions (outlined in the definition) which could otherwise explain symptoms (14). Notably, PTLD is a mechanistically neutral research definition and the term "post-treatment" refers to the patient's status of having been previously treated with appropriate antibiotics. In our experience, patients who meet this case definition are not uncommon in a Lyme disease referral practice in an endemic area, and represent ∼15% of patients referred for evaluation (11, 60).

The aim of this definition was to provide a framework for research, and to limit clinical heterogeneity in study populations (14). In research settings where reliability is methodologically

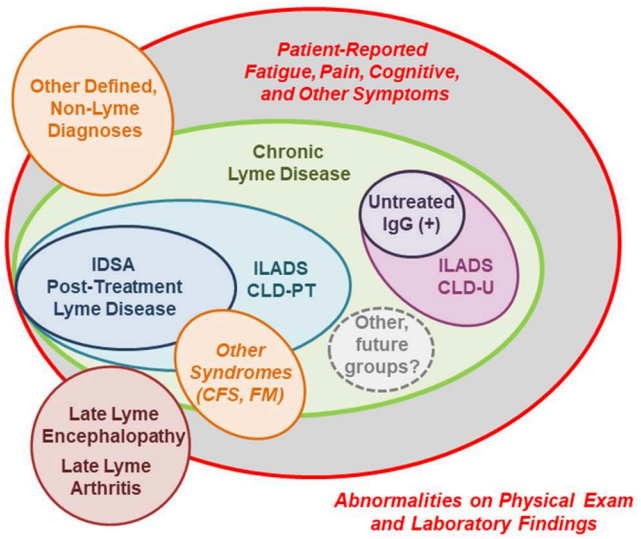

FIGURE 3 | A schematic of clinical- and research-defined patient subgroups among those with persistent symptoms associated with Lyme disease (14, 163, 174). The size of each patient subgroup is not meant to represent actual population frequency, as prevalence data is extremely limited. IDSA, Infectious Diseases Society of America; ILADS, International Lyme and Associated Diseases Society; CLD-PT, Chronic Lyme Disease-Previously Treated; CLD-U, Chronic Lyme Disease-Untreated; IgG, Immunoglobulin G; CFS, Chronic Fatigue Syndrome; FM, Fibromyalgia.

important in order to advance scientific understanding, it offers a way to identify a subset of those patients with on-going symptoms linked temporally to strong evidence of prior exposure to *B. burgdorferi* using the only tools currently available. As such, it is the most accepted and agreed-upon research subgroup of patients, despite having a symptom-defined clinical phenotype which lacks characteristic objective findings. While there are advantages to standardizing a research definition in this way, there are also limitations to the application of this definition in the clinical setting, as there are multiple pathways through which patients who may be suffering from on-going symptoms from Lyme disease may not meet these narrow criteria.

For example, a subset of these patients may have experienced initial delays in diagnosis, and/or misdiagnoses of their presenting signs or symptoms (43, 71, 176–179). The particular clinical difficulties in diagnosing EM (180–182), and/or a lack of EM or acute symptoms, remain on-going issues in community practice and can lead to significant delays in diagnosis and initiation of appropriate treatment. These scenarios have several important implications, as they not only increase risk for later persistent symptoms but they may also set patients on a clinical trajectory that never resembles the classic, textbook manifestations of Lyme disease. Furthermore, by the time patients have on-going, subacute symptoms, misdiagnosis or delays in diagnosis also increase the likelihood that patients will have been exposed to non-ideal antibiotics (43, 176). Partial treatment with non- or minimally-effective antibiotic regimens can explain the high rate of seronegativity and lack of seroconversion in this group. All of these factors hinder or obscure documentation of an initial episode in the medical record which meets CDC criteria for Lyme disease. Lastly, while the exclusion of patients with specific co-morbid conditions creates a high degree of illness specificity, it does not preclude the clinical reality that Lyme disease and its associated persistent sequelae often occur in the context of other pre-existing conditions.

Notably, the ILADS organization has also released a broader, more inclusive definition for CLD which encompasses those patients meeting the IDSA definition for PTLDS, as well as those with similar symptoms who would be excluded for lack of functional impairment or weaker evidence for prior Lyme disease (163). The ILADS CLD definition also differs from the IDSA's PTLDS definition in that it distinguishes between previously treated (CLD-PT) and untreated (CLD-U) presentations, and specifies on-going *B. burgdorferi sensu lato* infection as the underlying illness mechanism for both. However, ILADS CLD is a symptom-based definition and therefore ongoing infection is difficult to assess in individual patients with no currently available biomarker test, and given the large degree of symptom overlap between CLD and other illnesses.

Another unique subgroup of patients are those with only persistent, patient-reported symptoms similar to those reported in PTLD, a positive immunoglobulin G antibody response supporting prior exposure to *B. burgdorferi*, and no history of treatment for Lyme disease. Patients with this clinical presentation represented ∼6% of those referred for possible Lyme disease in our retrospective chart review (11). As these patients lack classic, objective signs of Lyme disease, they are often characterized as having ambiguous evidence of *B. burgdorferi* infection (174). However, this group may represent an interesting, intermediate clinical phenotype between archetypal Lyme disease and PTLD, as these types of presentations may occur before the onset of late Lyme arthritis, during which time objective manifestations may be transient (10).

Finally, given the lack of an available biomarker, there will always be a subset of patients with missed diagnoses of other diseases and conditions with similar symptoms, who initially seek evaluation for CLD with non-specific symptoms (11, 90, 91). This may include patients with other defined metabolic, inflammatory, neoplastic, or infectious diseases which can be differentiated by laboratory testing. It may also include patients who meet clinical criteria for syndromes such as fibromyalgia or chronic fatigue syndrome, which also have a high burden of non-specific symptoms such as fatigue and pain and lack clinically available biomarkers. These syndromes are commonly diagnosed, depending on the type and geographic location of the practice, in referral clinics among patients both with and without evidence for prior Lyme disease in their history (90, 91). Similarly, persistent symptoms in Lyme disease are sometimes attributed to misdiagnoses of common, chronic co-morbidities. Although this scenario does present an added degree of clinical complexity, it is important to consider the possible co-occurrence of two disease processes and the role of interaction between the two.

CURRENT CHALLENGES AND FUTURE PRIORITIES

Patients with PTLD represent a substantial burden to the United States health care system. In a large, health insurance claims analysis of 47 million members, estimated total direct medical costs from Lyme disease were between $712 million and $1.3 billion per year, with a significant portion of these specifically due to PTLD-related costs (183). The same study found that the adjusted odds of any PTLD-related symptom diagnosis following Lyme disease was 4.77 higher than age-, sex-, enrollment year-, region- and payer type-matched controls without Lyme disease, and that those patients with Lyme disease who went on to have at least one PTLD symptom had over twice the average total health care costs as those who did not (183). These cost estimates do not reflect additional indirect, non-medical, and lost productivity costs to patients, which may be substantial in a population with a chronic and significant illness impact on quality of life (80, 153, 184). Novel preventative approaches to reduce incidence of new Lyme disease cases, as well as physician and community educational interventions to increase awareness and reduce diagnostic delays and misdiagnosis, are needed to reduce costs and improve patient outcomes.

In a 2010 survey of primary care physicians in Connecticut, 50% responded that the did not "believe" in CLD, however almost all of the remainder (48%) responded that they were undecided or unsure (185). Despite the impression given that very few reputable physicians "believe" in CLD, many physicians and public health faculty acknowledge a real problem that is not just being driven by a small group of patients, physicians and advocacy groups (186). Given the wide variety of prescription, over the counter, and alternative treatments still currently being prescribed by physicians or sought independently by patients, there is a need to rigorously test new evidence-based therapeutic options for patients with persistent symptoms (187). Physicians would be aided greatly by illness biomarkers and effective tests of cure, as two-tier serology alone cannot be used in the post-treatment period for this purpose. However, such progress rests upon basic and translational scientific advancements in elucidating the biologic mechanisms of disease in this population.

Finally, only a handful of qualitative studies have been conducted which address the lived experience of patients with persistent symptoms (42, 153, 188–190). However, it is evident from this small body of literature that a key component of this experience is an often complex and protracted interaction with the health care system. Notably, patient frustration, financial burden, and "a long road to diagnosis" (189) are characteristic in the community practice of medicine (188). These factors are compounded by an immediate need to re-negotiate physical and social identities to the "new normal" of chronic illness, often without the same level of medical support and certainty afforded patients with non-contested conditions (153). We hypothesize that increased validation of the illness experience will improve interactions with the health care system and may also have under-appreciated downstream effects on patients' quality of life, coping, resilience, and even the physical burden of disease (191). An understanding of the ways in which the historical social construction of this illness and interactions with the health care system itself may contribute independently of, but in parallel to, biologic disease processes, is a final, important component of a multidisciplinary approach to alleviate patient suffering (192).

CONCLUSIONS

It is likely that the number of new Lyme disease cases will continue to increase in the coming decades, and consequently so will the number of patients with the variety of clinical presentations described in this article as having persistent symptoms. These symptoms are significant for the magnitude of their impact on the broader health care system, as well as on the quality of life of individual affected patients. Although much progress has been made to characterize and understand the most common manifestations of Lyme disease in the almost 50 years since it was first identified, many fundamental questions surrounding persistent symptoms remain unanswered. Beyond the uncertainty and the controversy, real opportunity exists for scientific insight into not just Lyme disease but also the increasing numbers of patients with unexplained symptoms and syndromes for which modern medicine does not currently offer explanation or treatment.

The identification of patient subgroups is an important way to address these questions and understand the heterogeneity of this patient population. PTLD is one such defined sequelae of Lyme disease which exists within the broader universe of patients with persistent symptoms. Irrespective of the underlying pathophysiology of the illness, it is a useful tool which can be operationalized in research settings where specificity and standardization is essential. Importantly, it can be used as a starting point to move the field forward scientifically, and eventually to understand other clinical subgroups where the established link to prior Lyme disease may exist but is less firm. Despite significant challenges, there is a critical need to develop and refine scientifically rigorous, multidisciplinary means of engaging with these more complex and controversial presentations of Lyme disease.

AUTHOR CONTRIBUTIONS

AR and JA contributed to the organization and design of the manuscript, performed literature reviews, participated in drafting sections of the manuscript, and in revising, reading, and approving the final submitted version.

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Conflict of Interest: JA has been granted a United States patent on the use of the T-cell chemokine CCL19 to identify patients at risk for PTLD who may potentially benefit from further treatment.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Borrelia miyamotoi: 43 Cases Diagnosed in France by Real-Time PCR in Patients With Persistent Polymorphic Signs and Symptoms

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Background: *Borrelia* species are divided into three groups depending on the induced disease and the tick vector. *Borrelia miyamotoi* is a relapsing fever *Borrelia* but can induce symptoms related to Lyme disease. Discovered in 1995, it is found in ticks around the world. In France, this species of *Borrelia* has been isolated in ticks and rodents, but was not yet observed in humans.

Objective: The aim of the study was to look for *B. miyamotoi* in symptomatic patients.

Methods: Real-time PCR was performed on 824 blood samples from patients presenting symptoms of persistent polymorphic syndrome possibly due to tick bite, a syndrome recognized by the French Authority for Health, which is close to the post-treatment Lyme disease syndrome. PCR was also performed on 24 healthy control persons. The primers were specifically designed for this particular species of *Borrelia*. The sequence of interest of 94 bp is located on the *glpQ* gene. Sequencing of amplification products, randomly chosen, confirmed the amplification specificity. To better investigate cases, a clinical questionnaire was sent to the patients PCR-positive for *B. miyamotoi* and to their physician.

Results: This search revealed a positive PCR for *B. miyamotoi* in the blood from 43 patients out of 824 (5.22%). PCR was negative in all control persons. A clinical chart was obtained from 31 of the 43 patients. A history of erythema migrans was reported in five of these 31 patients (16%). All patients complained about fatigue, joint pain and neuro-cognitive disorders. Some patients complained about respiratory problems (chest tightness and/or lack of air in 41.9%). Episodes of relapsing fever were reported by 11 of the 31 patients (35.5%). Chilliness, hot flushes and/or sweats were reported by around half of the patients. *B. miyamotoi* may not cross-react with *B. burgdorferi* serology.

Conclusion: This study is the first to detect *B. miyamotoi* in human blood in France. This series of human *B. miyamotoi* infection is the largest in patients with long term persistent syndrome. Our data suggest that this infection may be persistent, even on the long term.

Keywords: *Borrelia*, *Borrelia miyamotoi*, real-time PCR, borreliosis, Lyme disease, relapsing fever, post-treatment Lyme disease syndrome

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INTRODUCTION

Spirochetes of the genus *Borrelia* are divided into three major groups according to the vector and/or the pathology they can cause. Bacteria of the first group such as *B. duttonii* and *B. hermsii* are responsible for relapsing fevers, transmitted by soft ticks (*Argasidae*). Bacteria of the second group, such as *B. burgdorferi* and *B. afzelii* are the agents of Lyme disease, transmitted by hard ticks (*Ixodidae*). The third group includes species phylogenetically close to species of the first group, but transmitted by hard ticks, including *B. theileri* affecting cattle, *B. lonestari* affecting deer and *B. miyamotoi* affecting rodents (*Apodemus argenteus*, *Apodemus flavicollis*, *Myodes glareolus*, *Peromyscus leucopus*) and birds (*Cardinalis cardinalis*, *Parus major*, *Carduelis chloris*), which serve as intermediate reservoirs before humans (1–3).

Lyme disease, or Lyme borreliosis, is the most common tick-borne disease in the northern hemisphere. In Europe, the bacteria belonging to the complex *Borrelia burgdorferi sensu lato* (*B. burgdorferi s.l.*) are transmitted by the ticks of the genus *Ixodes*. The geographical distribution of Lyme disease is linked to that of the vector, mostly found in cool and humid habitats, such as forests. In France, the incidence of the disease varies according to the region studied, increases with years and is now observed on the whole mainland territory with an incidence of 104 cases per 100,000 inhabitants in 2018 (4, 5). However, the lack of physicians' obligation to report cases of Lyme disease makes difficult to determine its precise incidence and location. Furthermore the tick bite is often unnoticed by the patient. The primary stage of the disease is characterized by erythema migrans, a specific sign but not constant. Patients presenting with later stage of Lyme disease suffer from subjective or non-specific polymorphic signs and symptoms which may persist after the end of currently recommended antibiotic treatments. In most of the cases, there is asthenia, possibly disabling, with pain which may be localized in joints, muscles, bones or of neurologic origin. Pain is often migrating. Many patients complain about neurocognitive disorders. Most of the patients present with objective signs from different organs or systems (neurologic, rheumatologic, cutaneous, cardiac, visual…) but these signs are not specific and may be observed in other diseases. Lyme disease serology may be negative (6). Thus, physicians lack accurate diagnostic tests to better investigate the possible causes of these nonspecific syndromes. To further complicate the issue, it has been shown that some of these patients may suffer from other co-infections due to bacteria or parasites such as *Babesia*. Different names have been proposed to define these signs and symptoms, often mentioned as "post-treatment Lyme disease syndrome," PTLDS (7). In France, the denomination recognized by the High Authority for Health (Haute Autorité de Santé, HAS) in the official French Recommendation of Good Practice (June 2018) is "persistent polymorphic syndrome possibly due to a tick bite," (SPPT) (8). The difference between SPPT and PTLDS is that a diagnosis of Lyme disease has not to be proven and patients may have not been treated. It is now established that various species of *Borrelia* may be isolated from humans. *Borrelia miyamotoi*, discovered more than two decades ago, has been isolated from ticks and from patients in various regions of the world. Its real incidence in populations is not yet established.

B. miyamotoi was first described in 1995, isolated from ticks of the genus *Ixodes persulcatus* (9). Later, it was also observed in other tick species such as *I. scapularis*, *I. Pacificus*, and *I. ricinus* (10). Its DNA has shown similarities with other *Borrelia* species. It was named *Borrelia miyamotoi* sp. nov. (reference strain: HT31) and has been first classified with the *Borrelia* involved in relapsing fevers (9). However, further studies have shown that *B. miyamotoi* could provide in some patients signs and symptoms similar to Lyme disease. In 2011, a Russian team highlighted for the first time the presence of *B. miyamotoi* in humans. A large proportion of patients showed signs and symptoms similar to those caused by *B. burgdorferi s.l.*, including fever, headache, myalgia and arthralgia (11). The authors also found a high incidence of *B. miyamotoi* in the study area. Infections with *B. miyamotoi* seemed more severe than those observed with *B. burgdorferi* or *B. garinii*. In a study by Lee et al. (12), a highly conserved 357-bp segment of 16S rDNA gene of *B. burgdorferi s.l.* plus the correspondent 358 bp-segment of *B. miyamotoi* were amplified by nested PCR (single pair of primers). Amplicons were used as templates for direct Sanger DNA sequencing. This technique allowed, in winter, to detect spirochetemia in 14 patients. Among these, the bacterium involved was *B. miyamotoi* in four cases and a combinaison of *B. miyamotoi* and *B. burgdorferi* in one case. In immunocompromised patients, *B. miyamotoi* infection caused meningoencephalitis in the United States and in Europe in the Netherlands (13, 14). In France, the first study on *B. miyamotoi* was carried out in 2014 on ticks and rodents (15), demonstrating that 3% of the ticks and 5.55% of the rodents were infected with *B. miyamotoi*. Strain sequencing showed the same genotype not only in ticks, rodents but also in one Dutch patient reported by Hovius et al. (14). In Japan the same year, two publications showed that *B. miyamotoi* could be present in patients presenting with signs and symptoms suggesting Lyme disease (16, 17). Subsequently, *B. miyamotoi* has also been detected in other European countries such as Belgium and England (18, 19). In a study conducted in New York state using multiplex real-time PCR on 796 clinical specimens (blood and CSF), *B. miyamotoi* was found in eight cases (20). The frequency of *B. miyamotoi*, as a human pathogen, as well as the severity of some related signs and symptoms such as meningoencephalitis, make prevention, diagnosis and treatment of this infection essential (21). Furthermore, *B. Miyamotoi* may be resistant to some antibiotics such as amoxicillin, at least *in vitro* and has the ability to bypass the body's immune mechanisms, such as the complement by means of a surface protein, a factor H-binding protein, termed CbiA (complement binding and inhibitory protein A) (22–24). The local immune response is influenced by the tick, which secretes a multitude of immunosuppressive salivary factors that target the organism defense molecules. The subsequent immune reaction is delayed or incomplete thanks to the intervention of glycoproteins, called "evasins," which will bind to the chemokines secreted by the host, inhibiting their actions (25). A known problem of infections caused by (some, if needed) strains of group *Borrelia s.l.* is

the reappearanceor persistence of signs and symptoms after a classical treatment (26, 27). Recently, a study conducted in Russia, confirmed the presence of *B. miyamotoi* in 70 of 473 patients at the early stage of signs and symptoms occurring after a tick bite (28). This study showed that the median time for detection of *B. miyamotoi* in blood was 4 days after inoculation. No human case of *B. miyamotoi* has been described in France yet.

The purpose of this study, carried out in a population different from that studied in 2018 by Karan et al. (28), was to look for *B. miyamotoi* in the blood of patients living in France and suffering from a persistent polymorphic syndrome possibly due to a tick bite (7, 8). In case of positive tests, we obtained a first approximation of the incidence of the infection. Due to numerous positive tests, the study was further completed with a clinical evaluation. A standardized questionnaire was sent to the patients detected positive and to their physicians to obtain information about their medical history and clinical presentation.

PATIENTS AND METHODS

Patients and Samples

Blood samples were drawn from two groups of people. A control group was made up of healthy students of the University of Angers, not expressing signs or symptoms and located in a rural region of France ($n = 24$). The second group included patients, expressing signs and symptoms compatible with a persistent polymorphic syndrome possibly due to a tick bite and living in different regions of France ($n = 824$). These signs and symptoms included a range of conditions associated with fatigue, sleep disturbance, neurological/musculoskeletal pain, and cognitive dysfunction, lasting for at least 6 months. A questionnaire was used, including the main signs and symptoms usually observed during SPPT/PTLDS (7, 8).

Five milliliters of blood were collected by venous puncture in tubes with EDTA as anti-coagulant, before any antibiotic treatment and were sent in Vacutainer® K2 tubes.

TABLE 1 | Sequences of the primers used for *B. miyamotoi* PCR and sequences of housekeeping gene (GAPDH) primers.

Target	Gene	Primers	Probes
Borrelia miyamotoi	Glycerophosphodiester phosphodiesterase *glpQ*	F 5′ TGCACAATTATTTC CCAATCGA 3′ R 5′ TTCACTGAGACTTA GTGATTTAAGTTCAGT T 3′	
Human	*GAPDH*	F 5′ GAAGGTGAAGGTC GGAGT 3′ R 5′ GAAGATGGTGATG GGATTTC 3′	5′-6-FAMCAAGCT TCCCGTTCTCAG CC-BHQ1-3′

Selection of Primers

To allow the detection of *B. miyamotoi,* primers targeting the gene *glpQ* (Accession KU845211.1) of *B. miyamotoi* and framing of 94 bp portion of gene were used (29) (**Table 1**, **Figure 1**). The primers used in this study were derived from an existing publication by Reiter et al. who developed a new PCR approach for the detection of *B. miyamotoi* in ticks (30). Alignment of the sequence of interest of *B. miyamotoi* with the same portions of sequences in the genome of other *Borrelia* species confirms the specificity of the primers (**Figure 1**). In order to be more sensitive, a PCR simplex kit specific for *B. miyamotoi* was used, to avoid the loss of sensitivity common with multiplex kits.

Robustness of PCR Mixes

The portion of the *glpQ* sequence of *B. miyamotoi* was synthesized and introduced into a plasmid to obtain a control DNA and facilitate its multiplication. This control DNA was used to validate the amplification mix. Serial dilution of the plasmid was performed and amplified to determine the robustness parameters of the *B. miyamotoi* PCR kit: the limit of detection (LOD), the limit of quantification (LOQ), the repeatability and the reproducibility (**Table 2**).

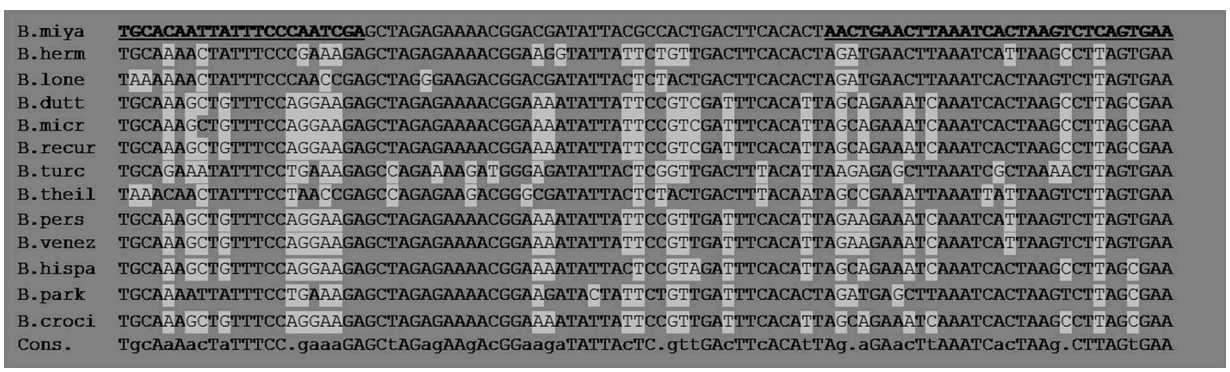

FIGURE 1 | Several sequences of the *glpQ* gene portion used for the detection of *B. miyamotoi* and belonging to other species of the recurrent fever group of *Borreliae* are aligned and compared. Bold and underlined: primers for the target *B. miyamotoi*. The *glpQ* gene, in the current state of the art and genome annotations has not been described in *Borrelia* species other than those of the recurrent fever group. Highlighted: the sequence differences of some recurrent fever *Borrelia* compared to that of *B. miyamotoi*. The following sequences are aligned: *B. miyamotoi* (D43777.1), *B. hermsii* (DQ855539.1), *B. lonestari* (AY368275.1), *B. duttoni* (DQ346787.1), *B. microti* (EU914144.1), *B. recurrentis* (DQ346781) .1), *B. turcica* (AB529430.1), *B. theileri* (KF569938.1), *B. persica* (AY530742.1), *B. venezuelensis (*MG651651.1), *B. hispanica* (GU357572.1), *B. parkeri* (MH704900.1), *B. crocidurae* (JX292940.1). The regions of the primers are very different between species.

TABLE 2 | Characteristics of the *B. miyamotoi* PCR kit.

	PCR Mix		LOD		LOQ		Repeatability	Reproducibility
	Tm	Mean efficacy	GU/PCR	GU/ml	GU/PCR	GU/ml	Mean CV	Mean CV
Borrelia miyamotoi	79.5°C	106.3%	12.5	1,041	18.8	1,567	0.85	1.22

Tm, melting temperature; LOD, limit of detection; LOQ, limit of quantification; GU, genome unit; CV, coefficient of variation.

DNA Extraction and Purification

The DNA was extracted without any prior treatment using 300 µl of whole blood with an equal volume of ADNucleis extraction buffer (5 M guanidium thiocyanate, 500 mM TrisHCL, 50 mM EDTA, 20% Tween 20, 20% Triton X-100, 750 µg proteinase K). After incubation for 20 min at 56°C and 15 min at 80°C, the extracted DNA was purified by means of silica magnetic beads and eluted in 250 µl of elution buffer (10 mM TrisHCl, pH 8.5).

Control of the Extraction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene as an internal control for PCR extraction and inhibition. The extracted samples were first checked with a PCR targeting the GAPDH gene. If the results of this PCR were consistent (Ct of GAPDH below 32), the samples were then analyzed for the other pathogens. The sequence of interest of GAPDH was inserted into a plasmid to be the *B. miyamotoi* target and this plasmid was used as a positive DNA for the validation of GAPDH primers and PCR mix as well as a positive control for subsequent PCRs. The primers used for GAPDH are described in **Table 1**.

Real-Time PCR

Real-time PCR was carried out in a total volume of 50 µl with a PCR mix containing ADNucleis PCR buffer (20 mM Tris-HCl, 10 mM NH_4SO_4, 10 mM KCl, 2 mM Mg2+, 0.1% TritonX-100, pH 8.8), 2 mM of each dNTP, 600 nM of each primer, 1 µl of Evagreen and 5 units of *Taq* polymerase ADNucleis. Twelve µl of extracted samples were amplified.

An initial denaturation step of 5 min at 95°C was followed by 42 cycles of 15 s at 95°C and 40 s at 60°C (hybridization-elongation). The dissociation curves were generated by a last step of 10 min with temperature increments from 75 to 95°C.

Quantification

Positive samples were quantified using a standard curve obtained by amplifying known and calibrated concentrations of control DNA of the desired targets. Quantification was obtained using the standard curve equation (Ct = a (Log10 [DNA]) + b) where "a" is the slope and "b" the intercept of the curve. The results were expressed in genome units (UG) per ml of blood.

Sequencing

The PCR results of some samples were verified by sequencing. A positive sample at the first PCR was amplified again in a second PCR with the same mix and primers. The product of this second PCR was then sent to an external provider for the sequencing of the obtained amplicons. Primers were supplied to the provider. The sequences obtained after sequencing were then compared to the expected sequence of the amplicon, which is specific of the target.

TABLE 3 | Lack of detection of *B. miyamotoi* in the healthy persons of the control group.

	PCR inhibition	Ct GAPDH values	Detection
FDC071	No	29.44	Not detected
MCM072	No	24.46	Not detected
MGA073	No	28.57	Not detected
MFA074	No	28.47	Not detected
FBF075	No	27.7	Not detected
MDW076	No	27.76	Not detected
FDT077	No	30.97	Not detected
MAJ078	No	28.29	Not detected
MMC079	No	28.81	Not detected
FMS081	No	28.08	Not detected
MSL082	No	31.28	Not detected
MMD085	No	31.55	Not detected
MPA088	No	30.57	Not detected
FVA089	No	29.98	Not detected
MGW092	No	31.17	Not detected
FDN093	No	29.15	Not detected
MBA094	No	31.87	Not detected
FFS095	No	28.32	Not detected
FBA096	No	28.7	Not detected
FGA098	No	28.89	Not detected
MACA101	No	26.54	Not detected
MLS103	No	30.83	Not detected
FLH105	No	31.77	Not detected
FLL106	No	28.44	Not detected
Positive control	No	22.83	Detected
Negative control	No	0	Not detected

B. miyamotoi was searched by qPCR on a control group of 24 healthy asymptomatic students. All analyzed bloods were negative.

RESULTS

Research of the Presence of *B. miyamotoi* by qPCR on Healthy Control Volunteers

The presence of *B. miyamotoi* was searched by qPCR on the control group of 24 healthy asymptomatic students. For all

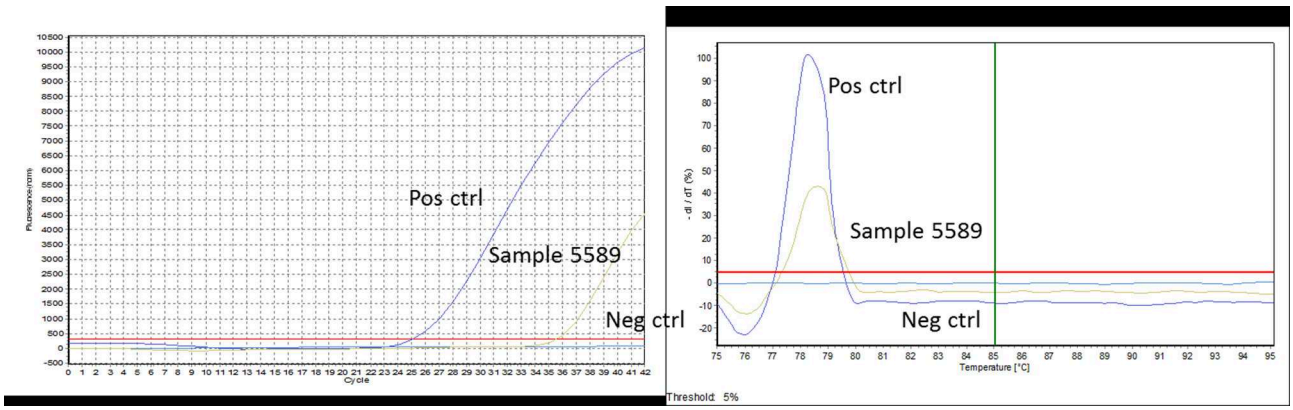

FIGURE 2 | An example of PCR curves obtained for sample 5589. The positive control well shows a Ct of 25 with a specific Tm of 79°C. Sample 5589 is amplified with a Ct value of 35 and the same specific Tm of 79°C. The negative control well shows no Ct and no Tm.

extracted blood samples, a Ct of less than 32 was detected for the GAPDH extraction control, which allowed further investigation. The results showed that none had *B. miyamotoi* infection (**Table 3**).

Results of Analyses on Symptomatic Patients

After the confirmation of the absence of *B. miyamotoi* in the group of healthy people, analyses were performed on the second group of symptomatic patients. Out of a total of 824 analyses, 43 samples were detected positive by qPCR for *B. Miyamotoi* (**Figure 2**), which corresponds to 5.22% of the patients. Of these 43 samples, *B. miyamotoi* could be quantified in 21 cases. In the remaining 22 cases, *B. miyamotoi* concentration was below the limit of quantification (**Table 4**).

Sequencing of the Amplicons Obtained

In order to confirm the specificity of the *B. miyamotoi*amplicons in the positive samples, eight positive and five negative samples (13 in total) were selected and subjected to DNA sequencing. Negative samples were sequenced in order to confirm that the readings retrieved were specific of positive samples and not a sequencing artifact. Negative samples had <20% similarity to the expected amplified sequence with no long runs of similar nucleotides (**Figure 3A**), while all positive samples showed greater than 60% similarity, even up to more than 90% for some amplification results (91% for sample number), with high number of similar consecutive nucleotides (**Figures 3B,C**). Giving the shortness of the amplified sequence (94 bp), as for any sequencing, the beginning (and sometimes a few base pairs at the end) of the sequence is often not available (used by the primer which is not sequenced) and accounts for the fact that only 60% of some of the sequenced amplicons are read.

Clinical Charts of Symptomatic Patients

Among the 43 patients with a positive PCR, 31 filled the questionnaire with their physician. Out of the 31 reported cases, 11 had their place of residence in Bretagne or the Loire Valley (West); the others were spread throughout the territory with a slight predominance for the South-West and the Rhône-Alpes region (South-East).

The duration of signs and symptoms divided the patients into two groups. For six patients, the duration of signs and symptoms was less than one year, while for 25 patients, signs and symptoms persisted on the long term (**Table 5**). Two patients have been sick for almost 30 years, two other patients for at least 20 years, the remaining patients between 1 and 19 years.

The results of the Lyme borreliosis ELISA test (commercial tests, performed in city laboratories) which is based on three species of the *B. Burgdorferi s.l.* complex (*B. burgdorferi s.s.*, *B. afzelli, B. garini*), are negative for 19 patients (76% of 26 informed cases), doubtful in three cases, positive in three cases, and not informed in six cases. Western-blot was negative in nine cases (50% of 18 informed cases), positive in nine cases (including three formerly positive and one doubtful with previous ELISA test). For 13 patients, Western-blot was not performed (eight cases) or no result was informed (five cases).

Erythema migrans, a sign specific for Lyme disease, was not frequent (**Table 5**).

Other recorded clinical signs and symptoms are reported in **Table 5**. Asthenia was constant and was usually happening quite abruptly, corresponding to a change of life for patients, in their personal, professional and sport activities. The asthenia intensity was graded with a 0–5 scale, and reported as "moderate" (score of 1–3) or "strong" (score of 4 or 5). The cephalalgia intensity was graded with a 0–5 scale, and reported as "moderate" (score of 1–3) or "strong" (score of 4 or 5). Some patients with neurocognitive disorders were unable to answer questions correctly. In these cases, it is their family members or relatives who answered for them.

A significant proportion of patients experienced signs suggesting thermoregulation disorders, including episodes of relapsing fever, an interesting fact since *B. miyamotoi* belongs to a group responsible for relapsing fever.

TABLE 4 | Results and quantification of samples detected positive for *B. Miyamotoi*.

N	ADNucleis ID	PCR results	Ct	Tm (°C)	Quantification GU/PCR	Quantification GU/ml	Comments
5	6107	Detected (LOD)	36.87	78.3	NA	NA	Detected but not quantifiable
7	5557	Detected (LOD)	36.74	78.6	NA	NA	Detected but not quantifiable
8	5113	Detected (LOD)	35.73	78.6	NA	NA	Detected but not quantifiable
9	5914	Detected	34.76	78.8	4.0E+01	2.79E+03	–
10	6072	Detected (LOD)	39.4	79	NA	NA	Detected but not quantifiable
11	6129	Detected (LOD)	39.39	79	NA	NA	Detected but not quantifiable
12	6273	Detected (LOD)	36.91	78.4	NA	NA	Detected but not quantifiable
13	6591	Detected	25.92	79	2.4E+04	1.68E+06	–
14	6594	Detected	31.59	78.6	1.5E+04	1.07E+06	–
15	6864	Detected (LOD)	33.82	78.2	1.99E+00	1.38E+02	Detected but not quantifiable
16	6784	Detected (LOD)	32.85	78.3	4.08E+00	2.83E+02	Detected but not quantifiable
17	7086	Detected (LOD)	37.22	78.5	2.15E+01	1.49E+03	Detected but not quantifiable
18	6527	Detected	34.26	79.5	2.7E+03	1.89E+05	
19	6749	Detected	29.91	79.7	4.58E+04	3.18E+06	–
20	6213	Detected (LOD)	39.66	79	NA	NA	Detected but not quantifiable
21	6630	Detected (LOD)	38.19	78.5	1.13E+01	7.88E+02	Detected but not quantifiable
22	6362	Detected	34.41	79.5	2.5E+03	1.71E+05	–
23	5815	Detected	30.13	79.1	4.0E+04	2.76E+06	–
24	6585	Detected	34.9	79	1.8E+03	1.25E+05	–
25	7147	Detected	36.49	78.2	9.47E+02	6.58E+04	–
26	6136	Detected (LOD)	37.52	78.4	NA	NA	Detected but not quantifiable
36	6235	Detected (LOD)	39.02	79.5	NA	NA	Detected but not quantifiable
37	6228	Detected (LOD)	35.76	79	NA	NA	Detected but not quantifiable
38	6231	Detected (LOD)	36.56	79	NA	NA	Detected but not quantifiable
39	6301	Detected (LOD)	38.53	79	NA	NA	Detected but not quantifiable
40	6407	Detected (LOD)	38.91	78.7	NA	NA	Detected but not quantifiable
41	6596	Detected	34.01	79.5	3.2E+03	2.22E+05	–
42	5589	Detected	34.13	79.5	3.0E+03	2.06E+05	–
43	6600	Detected	34.53	79.5	2.3E+03	1.59E+05	–
44	6603	Detected	34.61	79.5	2.2E+03	1.51E+05	–
45	6524	Detected	37.98	78.9	2.4E+02	1.69E+04	–
47	6615	Detected (LOD)	36.51	79	NA	NA	Detected but not quantifiable
48	6734	Detected	28.73	78.5	9.9E+04	6.84E+06	–
49	6735	Detected	33.04	79	6.0E+03	4.17E+05	–
50	6733	Detected	31.49	79.1	1.6E+04	1.14E+06	–
51	6985	Detected (LOD)	34.04	78.2	1.69E+00	1.17E+02	Detected but not quantifiable
52	6992	Detected (LOD)	33.25	78.3	3.03E+00	2.11E+02	Detected but not quantifiable
53	7159	Detected	37.13	78.5	6.24E+02	4.33E+04	–
54	7160	Detected	37.43	78.5	5.13E+02	3.56E+04	–
55	6578	Detected	33.99	79.5	7.0E+01	4.87E+03	–
56	6576	Detected (LOD)	35.64	79.5	NA	NA	Detected but not quantifiable
60	7099	Detected (LOD)	34.28	78.3	1.41E+00	9.81E+01	Detected but not quantifiable
61	5430	Detected	32.91	78.5	2.3E+02	1.27E+04	–

In 43 blood samples, B. miyamotoi was detected, i.e., 5.22% of the samples analyzed. For 22 samples, the detection was inferior to the limit of quantification of the B. miyamotoi PCR kit thus these samples could not be quantified. These 22 samples are said to be positive in limit of detection (LOD). For 21 samples, quantification was possible. The melting temperature of the B. miyamotoi positive control is 79°C and the tolerance range for the B. miyamotoi positive Tm is 79 ± 1.5°C. The melting temperatures of the amplicons coming from the detected samples are between 78.3 and 79.5°C. These amplicons are due to specific amplifications produced by the B. miyamotoi primers.

DISCUSSION

B. miyamotoi belongs to the relapsing fever group of pathogenic *Borrelia*. Rather few cases of *B. miyamotoi* infection were identified in humans. There is a debate about the clinical picture of the disease. It can be responsible for relapsing fever; however some clinical cases were more similar to Lyme borreliosis, including some cases with erythema migrans. The present study, conducted in France, is the largest case series of *B. miyamotoi* infection detected in patients suffering from long term persistent

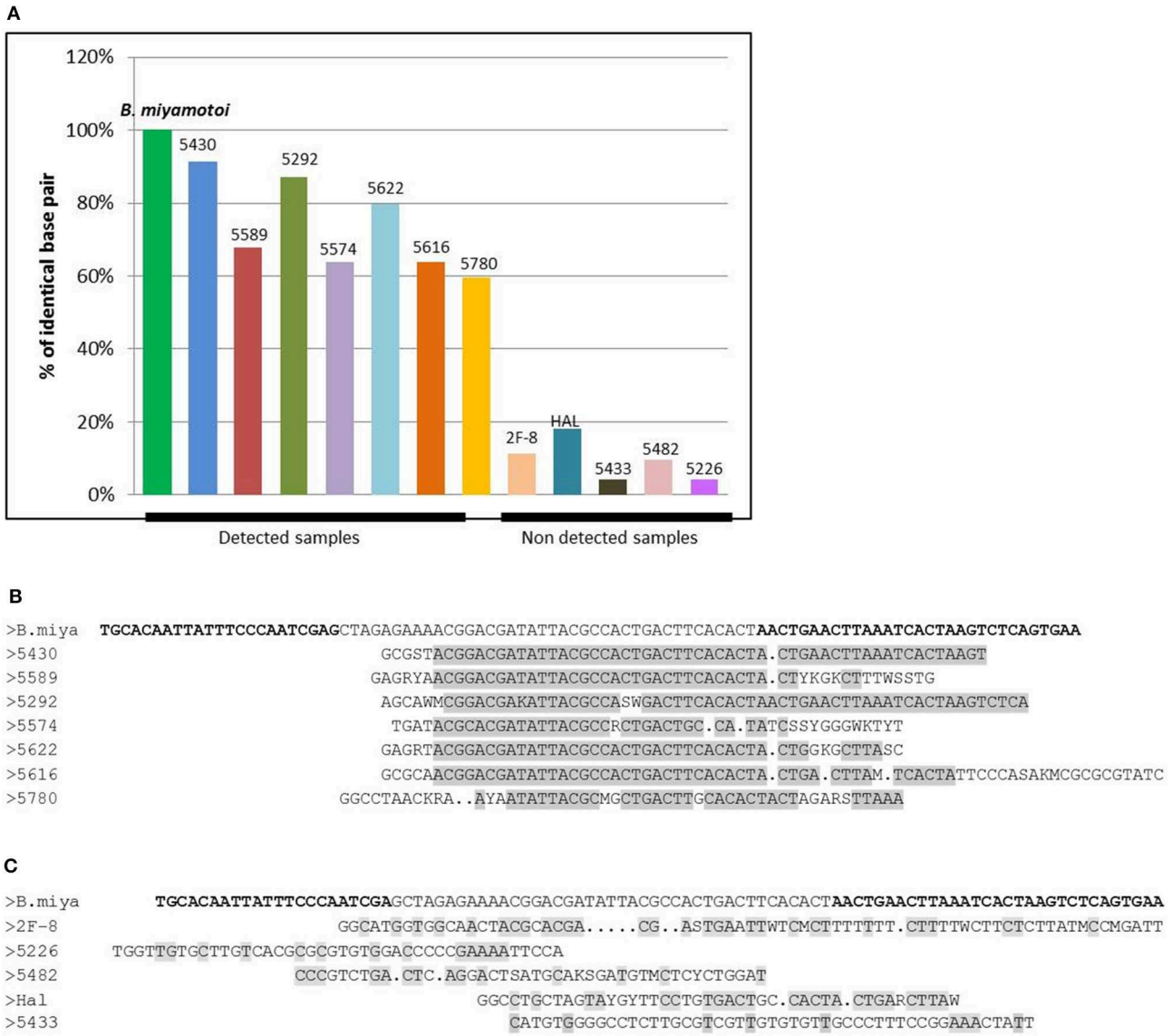

FIGURE 3 | (A) Graph of the percentage of identical base pairs for the detected and undetected samples for *B. miyamotoi* PCR. The percentage of identical sequenced bases is >60% for the detected samples which display long runs of identical bases **(B)** and <20% for the samples not detected in PCR with no long runs of identical nucleotides **(C)**. The sequence of interest of *B. miyamotoi* is a very short sequence (94bp). When sequencing small sequences, the first bases may not be recognized by the sequencer because of the brevity of the sequence, which explains why for some positive samples, sequencing only returns 60% of the bases of the sequence.

syndrome. It complements the Russian study by Karan et al. published in 2018 (28), which was conducted at the early stage on patients presenting with acute symptoms after a tick bite. The results of both studies suggest that *B. miyamotoi* is more frequent in humans than previously thought. We provide a gross description of the clinical signs and symptoms and the duration of the disease. However the lack of power of the clinical part of the study does not allow a definite conclusion about a precise clinical description. *B. miyamotoi* has a particular position in the genus *Borrelia*. No serology is available in routine. The sensitivity of PCR for the species belonging to the *B. burfdorferi s.l.* complex appears to be rather low, especially in blood. The sensitivity of PCR for *B. miyamotoi* is not known. The species *B. miyamotoi* may also suffer from a deficit of detection. PCR may become a useful means for the detection of *Borrelia,* amplifying the *fla* gene for flagellin, specific of *Borrelia* species. The *fla* gene is present in all *Borrelia* species with several conserved portions between the different *Borrelia species*. The choice of the sequence of interest should depend on the chosen target i.e., *B. burgdorferi sensu stricto* or *sensu lato*. As the *fla* gene

is also present in the genome of *B. miyamotoi*, it is possible that *B. miyamotoi* could have been detected and included in the *B. Burgdorferi s.l.* complex. The use of a kit specific for *B. miyamotoi* target probably favored our isolation. *B. miyamotoi* is a species apart, pathogenic and probably non-commensal as suggested by the fact that the healthy students of the University of Angers are not infected, while being in a rural area rich in ticks.

As evidenced in the publication by Reiter et al. (30) and the sequence alignment, the sequence fragment used for the detection of *B. miyamotoi* in the blood of the French patients tested is strongly homologous to other European strains of *B. miyamotoi* found in patients (KJ847051.1, AB824855.1, AB824730.1), showing only two nucleotides differences between sequences; differences which does not affect detection by PCR (see **Figure 4**). The *glpQ* gene was chosen for its specificity as it is, to the best of our current knowledge, only present in *B. miyamotoi* strains. Thus, the detection of a said gene is indicative of the presence of the pathogen. Additional genes often show lack of specificity, especially the 16S RNA or *flaB* genes, which are highly conserved in all *Borrelia* species, including those of the relapsing fever group. Indeed, sequencing of really short PCR fragments is often challenging as the first 20 or so nucleotides (the primer) are already "lost" due to the intrinsic nature of sequencing which does not "read" the primer used. Our claim is not with the percentage of similarity *per se*, our claim is in the consecutiveness of those homologous nucleotides. Forty identical consecutive bases, even in a 94 bases long fragment, deriving from a gene is specific of a pathogen.

The sequencing carried out shows that the amplicon obtained by PCR corresponds, for more than 60% and up to 90% of the purine and pyrimidine bases, to the desired target sequence specific for the *B. miyamotoi* species.

During the study period, *B. miyamotoi* was found with a high frequency (5.22%) compared to the other *Borrelia* species, i.e., *B. burgdorferi s.l.* (including *B. burgdorferi s.s.* , *B. garinii, B. afzelli, B. bissettii, B. spielmani, B. kurtenbachi*): 0.73% and *B. hermsii*: 0.36% (data not shown).

This pilot study, conducted in patients from various regions in France, suggests that *B. miyamotoi* infection could be more frequent in humans than previously thought and perhaps more frequent than other species of *Borrelia*, especially those classically responsible for Lyme disease. The signs and symptoms of persistent polymorphic syndrome possibly due to a tick bite are close to those described as post-treatment Lyme disease syndrome. Erythema migrans was observed in 16.1% of the patients, but data are insufficient to rule out a previous infection with *B. burgdorferi s.l.* However the responsibility of *B. miyamotoi* in some cases of erythema migrans is probable since, in the study looking at the early stage of the tick-borne infection, 3% of patients with an erythema migrans had a positive blood PCR for *B. miyamotoi* (28). Our data suggest that the disease may be persistent, even on the long term and that this species of *Borrelia* may not cross-react with *B. burgdorferi* serology. Asthenia, joint pain, neurocognitive disorders and sleep disorders were reported by all patients. Episodes of relapsing fever were observed in 35.5% of the cases. A large prospective study is needed to further describe this infection in well-defined populations.

TABLE 5 | Clinical signs and symptoms of 31 patients* with a PCR, performed from a blood sample, positive for *Borrelia miyamotoi*.

	Number of patients (%)	Description	Number of patients (%)
Duration of signs and symptoms		Less than 1 year	6 (19.4)
		Long term**	25 (80.6)
Signs and symptoms			
Erythema migrans	5 (16.1)		
Asthenia	31 (100)	Moderate	10 (32.2)
		Strong	21 (67.8)
Joint pain (often migrating)	31 (100)	Moderate	9 (29)
		Strong	22 (71)
Neurocognitive disorders	31 (100)	Loss of concentration, attention, memory and/or speech	
Sleeping disorders	31 (100)		
Other pains		Myalgia	25 (80.6)
		Including muscle cramps	16 (51.6)
		Cephalalgia (strong)	20 (64.5)
Thermoregulation disorders and associated signs		Chilliness	18 (58)
		Hot flushes	16 (51.6)
		Sweats (mainly at night)	15 (48.4)
		Relapsing fever	11 (35)
Respiratory symptoms		Chest tightness/lack of air	13 (41.9)
		Dyspnea	6 (19.4)
Balance disorders/malaises		Repeated falls	3 (9.7)
		Repeated malaises	2 (6.5)
Visual disturbances		Amputation of the visual field	1 (3.2)
		Diplopia	1 (3.2)
Other neurologic disorders		Parsonage-Turner syndrome	2 (6.5)
		Multiple sclerosis	1 (3.2)
		Manic depressive psychosis	1 (3.2)

These 31 patients are those, among the 43 patients of the study, who fulfilled with their physician a questionnaire.

**For six patients, the duration of signs and symptoms was less than one year, while for 25 patients, average duration of signs and symptoms was 9 years, with a range from 1 to 30 years. Two patients have been sick for almost 30 years, two other patients for at least 20 years, the remaining patients between 1 and 19 years.*

In conclusion, among French patients suffering from a persistent polymorphic syndrome possibly due to a tick bite (SPPT), a syndrome close to post-treatment Lyme disease syndrome (PTLDS), 43 out of 824 (5.22%) had *B. miyamotoi*in their blood identified by specific real-time PCR, including 22 cases at the detection limit and 21 quantifiable cases. This is the first detection of this bacterial species in humans in France. Sequencing showed the specificity of the detected DNA as *B. miyamotoi*. This study highlights that the lack of detection of *B.*

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>Bmiya       TGCACAATTATTTCCCAATCGAGCTAGAGAAAACGGACGATATTACGCCACTGACTTCACACT
>KJ847051.1 TGCACAATTATTTCCCAATCGAGCTAGAGAAAACGGACGATATTACGCTACTGACTTCACACT
>AB824855.1 TGCACAATTATTTCCCAATCGAGCTAGAGAAAACGGACGATATTACGCTACTGACTTCACACT
>AB824730.1 TGCACAATTATTTCCCAATCGAGCTAGAGAAAACGGACGATATTACGCTACTGACTTCACACT

>Bmiya       AACTGAACTTAAATCACTAAGTCTCAGTGAA
>KJ847051.1 CACTGAACTTAAATCACTAAGTCTCAGTGAA
>AB824855.1 CACTGAACTTAAATCACTAAGTCTCAGTGAA
>AB824730.1 CACTGAACTTAAATCACTAAGTCTCAGTGAA
```

FIGURE 4 | Alignment of the *Borrelia miyamotoi* French strain with the other European *Borrelia miyamotoi* strains (KJ847051.1, AB824855.1, AB824730.1). The sequences show a single nucleotide difference that does not affect the PCR and the PCR efficiency.

miyamotoi is not due to the absence of this particular species of *Borrelia* in France, but rather because this species was not sought out. Clinical studies designed to evaluate the correlation of PCR results with clinical signs and symptoms should be done to better investigate patients suffering from persistent polymorphic signs and symptoms of unclear origin.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The human studies were reviewed and approved by the Comité de protection des personnes CPP SUD 9EST VI Clermont Ferrand, France. All patients and control persons provided written informed consent.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: MF is CEO of ADNucleis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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GENERAL COMMENTARY
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Commentary: *Borrelia miyamotoi*: 43 Cases Diagnosed in France by Real-Time PCR in Patients With Persistent Polymorphic Signs and Symptoms

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A Commentary on

***Borrelia miyamotoi*: 43 Cases Diagnosed in France by Real-Time PCR in Patients With Persistent Polymorphic Signs and Symptoms**

by Franck, M., Ghozzi, R., Pajaud, J., Lawson-Hogban, N. E., Mas, M., Lacout, A., et al. (2020). Front. Med. 7:55. doi: 10.3389/fmed.2020.00055

INTRODUCTION

Ixodes ticks are the vector of the *Borrelia burgdorferi* sensu lato complex causing Lyme borreliosis (LB) and of *Borrelia miyamotoi*, a relapsing fever *Borrelia* species causing *Borrelia miyamotoi* disease (BMD). The latter disease entity was first described in 2011 (1), and its clinical symptoms in patients in Asia, Europe, and the USA mostly consist of a flu-like illness (2). The recent Frontiers in Medicine article by Michel Franck et al. claims to have detected *Borrelia miyamotoi* DNA in 43 out of 824 French patients with a complex of non-specific symptoms lasting at least 6 months (3). However, we have serious doubts about the author's findings and conclusions. In this commentary, we describe evident shortcomings of this study and urge for a reconsideration of its interpretation and conclusions.

PATIENT DESCRIPTION

The paper describes a poorly characterized patient population: it is unclear to which institutions they presented and how they were included in this study. Furthermore, the inclusion criteria are not described. Blood was collected, but it is unclear when and where this was done and how these samples were processed. Finally, while clinical characteristics of 31 patients with positive *B. miyamotoi* PCR and available questionnaires were described, the authors omitted to describe the clinical characteristics of PCR-negative patients and controls.

PCR METHOD

The PCR that was performed was based on a single target (*glpq*), which was also present in the positive control and thus posing a risk for contamination, despite the necessary countermeasures and controls. Furthermore, the low number of negative healthy controls does not exclude the possibility of false-positives dominating the results in the studied patient group: the proportion of positive PCR findings in the patient group does not differ significantly from the healthy control group ($p = 0.63$, Fisher's exact test). Moreover, the median bacterial load described by Franck et al. was supposedly twenty times higher than in well-described patients with severe acute BMD (4). Thus, the PCR results presented in this paper appear to be at risk of representing contamination with either positive control or PCR amplicons. One obvious way to lower this risk would have been a second PCR targeting an independent target.

SEQUENCING RESULTS

Another way to demonstrate that the positive PCRs were not false-positives is sequencing. The authors sequenced eight out of 32 positive samples and performed sequencing on the same fragment that was used in the qPCR. The authors used a plasmid control as a positive control in their qPCR assays, which contains a 94-bp fragment of the *glpq* gene from a Japanese *B. miyamotoi* isolate (HT-31, AB900798). This small and conserved fragment is 40 bp long (minus the primers) and differs from the Western-European *B. miyamotoi* isolates in one nucleotide (position 26, **Figure 1**). As far as we know, all Asian (*I. persulcatus*-associated) isolates contain a Cytosine whereas all known West-European (*I. ricinus*-associated) isolates contain a Thymidine at that position (**Figure 1**). Also, 12 French *B. miyamotoi* isolates (GenBank accession numbers KJ425352–KJ425363) from an independent study (5) contain a Thymidine at position 26, two of which are depicted in **Figure 1**. Six from seven of the *B. miyamotoi* sequences from the French patients in the study of Michel Franck et al. contained a Cytosine at position 26, identical to their positive control and deviant from all known *glpQ* sequences in European (*I. ricinus*-associated) *B. miyamotoi* isolates (**Figure 1**). It is therefore likely that the authors have amplified their positive control as a contaminant in these patient samples. Our request to obtain materials to perform an independent PCR was denied with the argument that blood samples and even DNA extracts were no longer available.

INCONSISTENCY OF THE RESULTS WITH PREVIOUS STUDIES

The presented results appear to be in conflict with current knowledge on *B. miyamotoi* pathogenesis and disease manifestations: The patients included in this study had symptoms for at least 6 months, and out of 31 patients with a questionnaire available, 35 percent described relapsing fevers. It is unclear what exact pattern these patients described, how high the fevers were, how long this lasted, and whether other diagnoses were identified. Furthermore, in studies with PCR-positive well-described BMD patients, relapsing fever has only been described as a rare and temporary phenomenon limited by either the use of antibiotics or by time (not more than a couple of weeks) (1, 6).

DISCUSSION

Currently, the diagnosis of Lyme borreliosis but also other tick-borne diseases suffers from the poor diagnostic yield of serology during the early disease manifestations and the lack of sensitivity of PCR on blood and CSF. Although clinical diagnosis can indeed be very difficult, this has also created a large gray area and symptoms unrelated to LB have been attributed to the disease. The above has resulted in discontent

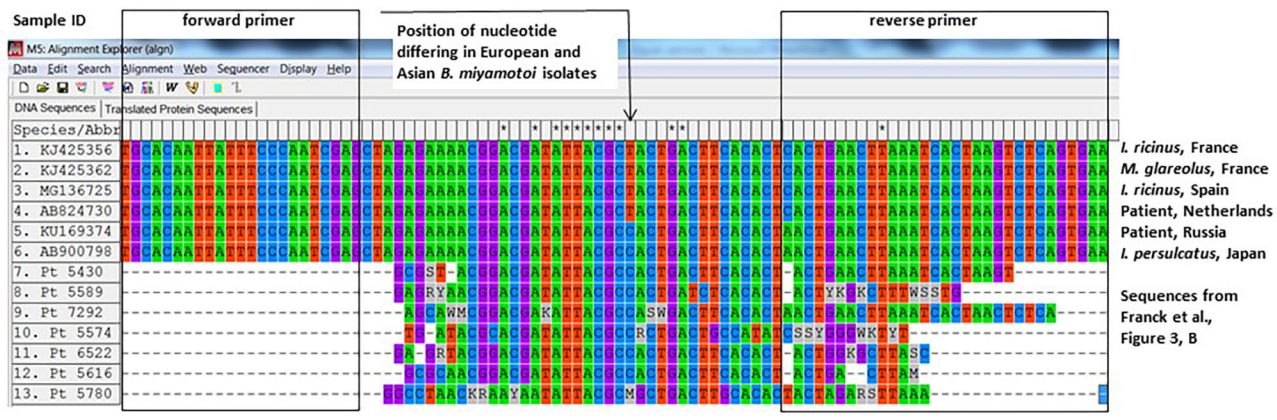

FIGURE 1 | Alignment of *glpq* sequences described in Franck et al. with previously described sequences from West-European and Asian *Borrelia miyamotoi* isolates.

within the public domain, both under- and over diagnoses, delay of proper therapy, and alleged failures of therapy. In contrast, for *B. miyamotoi* disease, the disease manifestations are thus far clearly defined, and PCR on blood appears to be a reliable tool to diagnose active infection. We have here outlined why the recent study by Franck et al., supposedly showing that long-lasting non-specific symptoms are associated with active *B. miyamotoi* infections, has too many shortcomings to redefine the clinical symptoms of BMD. In our opinion, their findings and conclusions should not have any implications for clinical decision-making.

AUTHOR CONTRIBUTIONS

AW, HS, AP, and JH have written the manuscript. HS and AP have performed the gene alignment, AP has generated the figure. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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An Overview of Tickborne Infections in Pregnancy and Outcomes in the Newborn: The Need for Prospective Studies

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Tick-borne infections are an ever-increasing issue internationally, many factors contribute to this including a changing climate. Pregnant women represent the single largest vulnerable group in populations due to a relative immune deficiency status. Infections in pregnant women have the added gravity of potential infection in the developing fetus which may have catastrophic consequences including death *in-utero* or lifelong debilitation. Currently there is a paucity of data surrounding tick-borne infections in pregnancy and long-term outcomes for mother and infant for conditions like Lyme disease and co-infections. At present there are no established international surveillance systems to identify and gain understanding of these infections in pregnancy. Furthermore, the removal of Congenital Lyme Disease from ICD-11 codes hampers dialogue and characterization of borreliosis in pregnancy and stifles future developments of this understudied domain. This review makes the case for further study and re-opening a dialogue of tick-borne infections in pregnancy.

Keywords: neurodegenerative diseases, congenital Lyme disease, autism, tick borne infections in pregnancy, vertical transmission

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INTRODUCTION

Approximately 17% of all infectious diseases are vector borne with just over 50% of the world's population at risk at any time to one of these diseases (1). Those spread by vectors within the Insecta kingdom include mosquitoes, ticks, and flies. The focus of this review is on tick borne infections in pregnancy.

Pregnant women represent the single largest vulnerable population in society. Infections in this group not only impact the mother but have the added gravity of impacting the unborn fetus during the most fragile time of human development and can result in catastrophic lifelong changes in the unborn and also intra uterine death. This risk is compounded by immune modulation in pregnancy with, reduced CD/CD8 cells, decreasing cytotoxic T cells, and a shift from Th1 to Th2 Helper T Cells all increasing susceptibility to infection (2, 3). Overall there is poor understanding of infection and treatment of infection in pregnancy. The complex interactions at the materno-fetal interface are poorly understood. The role of the placenta as both a protective barrier from infection but also from maternal immune recognition while also supplying the fetus with all the essential nutrition for human development needs much more research. The spectrum of disease severity and presentation of tick-borne congenital infections from classic and well recognized syndromes to insidious atypical presentations that emerge after delivery in the developing child will be hypothesized and discussed based on recent published data.

Perinatal outcomes following infections acquired during pregnancy can range from minor self-limiting illnesses, to pregnancy loss by spontaneous abortion, invasive fetal infection, and can sometimes result in congenital syndromes. The timing of fetal infection *in utero* may determine the extent of disease manifestations and the outcome to the unborn child. In essence infections during the first trimester during development and folding of the neural tube and early brain development can result in catastrophic developmental defects. Certain infections may predispose the pregnancy to preterm labor and pre-term delivery, with adverse outcomes secondary to prematurity. Other infecting organism can have a direct effect, there is also the aberrant host immune response to these invading pathogens, and subsequent immunological damage. Thus infections in pregnancy can have a wide variety of outcomes, depending on the timing of infection, the type of infection, the interaction between the infecting organism and the immune system, and indeed certain host factors.

Infections can additionally be transmitted in the peri-partum period from mucosal exposure, and post-partum through breast feeding; such infections may manifest themselves immediately or in the later post-partum period, or even later in childhood and indeed extending into adolescence and adulthood. This time delay makes it much more difficult to link the original congenital infection with delayed complications and ultimately adverse medical outcomes for the offspring.

TICK BORNE INFECTIONS IN PREGNANCY

Hard-shell tick-borne infections primarily affect northern hemisphere temperate climates but have been found on all continents including Australia. Epidemiologically with rising global temperatures diseases like *Lyme borreliosis* are rising in incidence in Europe and north America, as ticks have a longer feeding season (4). Beyond the issue of "global warming" humans live "closer" to animals, and changes in our planet have resulted in an increase in zoonoses worldwide. As more humans are being bitten and infected, a significant percentage of these humans are women of child-bearing age, and indeed some are already pregnant.

Lyme Disease

Lyme disease (LD) was first officially described in the State of Connecticut (Old Lyme), when a case series of children with juvenile arthritis were found to have spirochetal illness in 1977 (5). *Borrelia burgdorferi* was identified as the causative organism whose name is also now also used to describe a larger *Lyme borreliosis* complex (*Borrelia burgdorferi sensu lato*) which include *Borrelia burgdorferi sensu stricto, borrelia garinii, borrelia afzelii, borrelia miyamoti* amongst others. Vectors of Lyme are hard shelled ticks *ixodes scapularis* and *ixodes pacificus* in North America, *ixodes ricinus* in Europe and *ixodes persulcatus* in Asia. Ticks are also vectors for other disease like Ehrlichia, Rickettsiae, Bartonella, and Babesia, and often more than one infection can be spread by a tick at the same bite (called co-infections). Nymphal ticks that feed on small mammals and birds are the most transmissible of Lyme to humans. Infected ticks in endemic areas can have a wide range of prevalence's, ranging from 6-15% in Ireland, to over 50% in many EU countries and in the USA.

Vertical transmission of LD was first suspected in 1983 in a case that described arthritis in amother. Spirochetes were visualized on a blood film of the newborn who had presented with hyperbilirubinemia. However, no Lyme or syphilis serology was performed in this case (6), limiting conclusions. The first confirmed case with positive Lyme serology was described in 1985 in a 28-year-old mother who had acquired Lyme in the first trimester, who had a erythema chronicum migrans (ECM) rash, and delivered at 35 weeks. Symptoms consistent with LD developed in the mother post-delivery and her LD IFA was positive 1:128. The child died of congenital heart disease and autopsy showed spirochetes infiltrating the spleen, kidneys, and bone marrow, but were not found in cardiac tissue (7). A report by MacDonald successfully demonstrated *Borrelia burgdorferi* in the myocardium using an immunohistochemical technique (8).

In the following years a number of case reports present compelling immunohistological evidence of spirochetaemia in stillbirths where mothers had clinical and/or laboratory confirmed LD; confirming the vertical transmission of *B. burgdorferi* (8, 9). Evidence of clinical LD has been seen in infants in some instances: a 3 week old who developed a skin rash post-partum was found to have *B burgdorferi* isolated from biopsy of these skin specimens (10).

A case of neonatal LD was reported whereby Borrelia specific antibodies were discovered in the spinal fluid of an infant with documented neurologic dysfunction. The mother who had LD infection in her second trimester had been treated with oral antibiotics and was reported as being seronegative at the time of delivery (11). A case from Germany described an infant with neonatal onset of maculopapular skin rash, hepatosplenomegaly, anemia, and fever, followed by progressive multi-system manifestations including protruding eyes, bilateral knee arthritis, axillary and inguinal lymph nodes, growth impairment, and developmental delay. Elevated antibody titres against Borrelia were found in the child's serum; her mother, who had no clinical manifestations, also had positive ELISA titres (12). A case review of 19 women with LD in pregnancy reported adverse events in 5 cases of fetuses, suggesting the possibility of congenital LD (13).

Other suggestions of transplacental transmission pregnancy comes from studies of placental tissue tested post-delivery in mothers with LD; in one study of 60 mothers found to have antibodies against Borrelia, 5% had evidence of spirochetes in placenta tissue using silver stain. Two of 3 were PCR positive for *B burgdorferi* (14).

A study performed by Strobino et al. of over 2,000 women from an endemic region who 1had positive LD serology were compared to a Lyme negative cohort. Worse outcomes when comparing fetal deaths, pre term delivery and congenital abnormalities were not seen. Furthermore no risk of adverse outcomes was reported in women with reported tick exposure. In this study only 11 women had positive LD serology, 5 of whom had previously documented LD and who had received treatment (15). It is important to note that congenital defects in babies at 6-month follow-up was the only study marker of

adverse outcome in newborns. There was no direct detection testing of placentas or of cord blood of babies born to these seropositive women Longitudinal health monitoring, serial serologies in newborns was not performed. The authors reported "a statistically significant association between past miscarriages and history of tick-bite" and "a significant association between having had a tick bite within 3 years of conception and congenital defects." Authors also noted that the incidence of cardiac defects was twice as high in children born to mothers residing in towns with a high LD endemicity rate vs. low endemic areas. The authors also acknowledged that their study was underpowered and "the number of women was too small to draw conclusions about the risk of having a child with a congenital malformation if a woman is seropositive."

A recent review on congenital tick-borne diseases by Jasik et al. opines, "it is possible that B. burgdorferi has a high ability to penetrate mammalian placentae due to its ability of active movement, antigenic and morphological variation, and many other features, which causes diagnostic difficulties and problems. In cases of intrauterine fetal infections among patients with Lyme disease, symptoms are not homogeneous. This suggests that *B. burgdorferi* s.l. is transmitted trans-placentally and may play an important role in the spreading of these pathogens." Authors also acknowledge "the ability of long-term survival of *B. burgdorferi* s.l. in tissues and spreading of spirochetes in the body despite antibiotic treatment can contribute to intergenerational spread of Lyme disease" (16).

Most recently in 2018, Waddell et al. performed a systematic review of gestational LD and identified 59 cases between 1969 and 2017. Twelve cases report miscarriage or fetal death, 8 report new born death and 16 report other abnormalities post-delivery including syndactyly, respiratory distress, hyperbilirubinemia. One case described complete features of clinical and laboratory results consistent with vertical transmission of LD (17). They also summarized epidemiological studies comparing pregnant women in endemic areas with features or serology to non-Lyme pregnancies; their conclusion was that rates of adverse outcomes were not increased. There are discrepancies in the findings and interpretation of studies from the Waddell "systematic review" compared to other publications and reviews on this subject; questioning the accuracy of the term "systematic" in the title of their publication.

The literature on "Congenital Lyme" is at present incomplete due to lack of intensive investigations, and lack of longitudinal follow up of exposed infants, as has been done for another spirochete, syphilis. There is no doubt that congenital infection occurs with Borrelia; whether a congenital syndrome occurs as a result of this *in utero* infection remains to be further investigated.

Treatment of LD in pregnancy is complicated as doxycycline, the mainstay of treatment in non-pregnant adults, holds FDA class D in pregnancy due to disruption of teeth and bone during development. ca. Second line treatment with amoxicillin is advised in pregnancy, and recommendations suggest same treatment duration (18). Treatment of gestational LD has been associated with reduced adverse outcomes for the fetus (11%) vs. women not treated for infection in pregnancy (50%), which indicates some adverse outcomes for untreated gestational LD (17). A 2010 study authored by Lakos et al. reported adverse outcomes in parentally treated (12%), orally treated (31.6%) and of untreated women (60%) with LD during pregnancy (19). Some clinicians report preferential use of IV ceftriaxone 2G daily for 14 days for pregnant women with ECM, reporting a positive outcome in pregnant women and also good pregnancy outcomes (19–21).

Ehrlichiosis

Ehrlichiosis is characterized by two separate genetically linked organisms with similar clinical presentations; *Anaplasma phagocytophilum* and *Ehrlichia chaffensis*. Both are gram negative obligate intracellular organisms. *A phagocytophilum* causes Human Granulocytic Anaplasmosis (HGA) and *E. chaffensis* causes human monocytic ehrlichiosis (HME). Both organisms are spread by the hard shelled ixodes ticks similar to LD, *I. scapularis* in eastern and upper mid-western United States and *I. pacificus* in western United States.

HGA

HGA is clinically characterized by flu like illness, leukopenia, thrombocytopenia, transaminitis, raised alkaline phosphatase, and raised LDH, with symptoms following outdoor activity. Morulae and intracellular inclusion are characteristically seen within neutrophils on Wright or Giemsa stain. IFA with 4-fold increase in antibodies is the diagnostic test of choice but peripheral smear and serum PCR can be more sensitive in early disease, performed before initiation of antibiotics. Although poorly described in pregnancy some case reports have shown infections can be treated successfully (22). Cases of miscarriage have been reported in patients treated for HGA with doxycycline (23). Vertical transmission have been reported in one mother who had tick exposure one week prior to delivering (24), another case series also reports vertical transmission in 1 of 6 women, no cases were seen in individuals treated with either rifampicin or doxycycline. HGA appears to have a mild course in pregnancy with no major adverse outcomes seen (25).

HME

HME although closely linked to HGA has some distinct features that differentiate it from HGA. The vectors for this bacterium are the lone star tick and the amblyomma tick. IFA is unreliable as a diagnostic tool in this instance. Morulae are seen in monocytes but with low frequency 1–20%. Only one adverse outcome has been reported in pregnancy, where a mother developed appendicitis and was treated with doxycycline. Both mother and baby had good long term outcomes (26).

Babesiosis

Babesiosis, primarily caused by *Babesia microti* in humans is an intra-erythrocytic protozoal infection spread by Ixodes hard shelled ticks. When acquired patients are commonly co-infected with *Lyme borreliosis* and Anaplasma. In Europe *B. divergens* is the most common species, infection in humans is less commonly described compared to the US. Babesia is the most common transfusion related infection reported to the FDA (27). Clinical characteristics include fever, malaise, chills, jaundice,

conjunctival hemorrhage, organomegaly, mild to moderate haemolytic anemia, thrombocytopenia. Diagnostics include PCR and wright giemsa stain which displays a characteristic "maltese cross" appearance of tetrads of merozoites within red cells. IFA, serology and ELISA are also used in diagnosis. Severity of disease is dependent on level of immune competence and the disease can progress to heart failure, ARDS, liver failure, renal failure, splenic rupture, and can carry mortality up to 20%.

As pregnancy is a relative immunocompromising state severe Babesia infections in pregnancy have been seen. Furthermore cases of vertical transmission, although rare, have been described. A congenital syndrome of fever, thrombocytopenia and anemia requiring transfusion is plausible. In one review of 9 cases, 2 were occult infections in mothers also infected with LD (28). Babesia can mimic HELLP (hemolytic anemia, elevated liver enzymes, and low platelets) syndrome in pregnancy. Patients from endemic areas, or who may have had blood transfusions from endemic areas should be investigated for Babesiosis. The first line treatment is atovaquone and azithromycin for mild to moderate disease and intravenous clindamycin and quinine for severe disease (18). Cases of use of clindamycin and primaquinee as first line therapy for mild and moderate disease have been reported as they have better placental penetration and potentially could reduce transmission.

Tick Borne Encephalitis (TBE)

TBE is a neurotropic flavivirus spread by the same ticks as LD in Continental Europe and Asia. *I. ricinius* and *I. persulcatus* have the same small mammal reservoirs; TBE can also be spread by contaminated raw milk, particularly from goats (29, 30). The initial phase is characterized by non-specific viral prodrome, followed by a period without symptoms. A second phase after 4–5 weeks is characterized by neurological sequelae; meningitis, meningoencephalitis, radiculitis, myelitis, and paralysis. A case report of infection in the third trimester of pregnancy resulted in self-limiting illness with an uncomplicated spontaneous vaginal delivery. The TBE antibody was negative in the healthy neonate (31). No cases with evidence of vertical transmission have been seen. An inactivated vaccine is available; vaccine should be administered before pregnancy in those at risk; administration in pregnancy should only be considered when deemed necessary and an appropriate risk/benefit ratio is made (32).

Relapsing Fever

While tick borne Borreliosis tends to focus on Lyme disease specifically, relapsing fever borreliosis (RF) is a significant cause of morbidity worldwide (33). Within tick borne relapsing fevers, the main vectors are the "soft ticks" of the genus Ornithodoros; but some species are transmitted by the ixodid vectors or "hard ticks." Few studies have been done on this group of bacteria to further elucidate the interactions between host, tick, and pathogens. In pregnancy, it is claimed that relapsing fever Borreliosis may cause up to 10–15% of neonatal deaths worldwide (34).

RF borreliosis in pregnancy has a spectrum of complications; decreases in birth weight and preterm delivery in mild cases, or severe damage with miscarriage or neonatal death in severe cases (35, 36). One case report describes pregnant women with mild RF symptoms, but ultimately fatal outcome to the baby, who succumbed within 30 h of delivery (37). Recent mouse studies have shown RF infection of the fetus, can cause intrauterine growth retardation as well as placental damage and inflammation. Impaired fetal circulation causes spirochete and erythrocyte interactions as well as lowered maternal hemoglobin, in addition to direct invasion of the placenta (38). Further prospective studies in humans are needed to confirm the animal studies done to date.

Rickettsial Disease

Rickettsia are a genetically related group of intracellular coccobacillary proteobacteria that have a pan-global distribution and cause febrile illnesses of variable severity. They are spread through a number of vectors including ticks, lice, fleas, and mites. There are over 20 species that can be broadly separated into four groups; ancestral, spotted fever, typhus, and transitional (39). Fever and rash are common features which can make rickettsial diseases difficult to distinguish from other infections.

Publications of clinical outcomes in pregnancy are limited in general as with other vector borne infections and appear to be worse than in the general population.

Rickettsia Rickettseii

Rickettsia rickettseii is the most pathogenic species and causes Rocky Mountain Spotted Fever (RMSF), a febrile illness with mortality rates as high as 20–30% without treatment. In the United States the American dog tick, a hard-shelled tick, *Dermacentor variabillis* in central and eastern states and *Dermacentor andersoni* in Western United States are the most common vectors. This infection is also endemic to other western hemisphere countries; Canada, Colombia, Brazil, Argentina, Costa Rica, Panama, and Mexico (40). A classical triad of fever, headache and rash is only present in around 60–70% of patients by week 2 post inoculation. Malaise, nausea, vomiting, abdominal pain are also features of early infection with mean onset of symptoms of 7 days. It can be missed in patients especially in pregnancy as other more common infections are suspected. The rash classically starts as a blanching macular rash at wrists and ankles and progresses to a non-blanching petechial rash that can become more confluent and progress to purpura. Occasionally progression to peripheral gangrene necessitates amputation. Disease progression can be severe within days of onset and can result in multi organ dysfunction, hepatomegaly, confusion, meningismus, pneumonia, and disseminated intravascular coagulation.

Studies in pregnant women are limited but the disease does not appear to be more severe in pregnant women. Vertical transmission has not been described. A case series of 4 patients in Mexico treated with doxycycline had negative outcomes. All of the mothers survived and one child born by SVD at 36 weeks had an uncomplicated course. Three women in the first trimester had spontaneous abortions (41). The authors identify 10 patients in the literature including their four patients. Doxycycline was used in 5 cases, chloramphenicol in 3 and amoxicillin in 2. Maternal fatality occurred in 3 cases, one was complicated by

amputation of digits due to gangrene and the remaining cases were uncomplicated. Three neonates died post-partum, three miscarriages, one neonate had transient hyperbilirubinemia, and three uncomplicated outcomes. In the two cases where amoxicillin was administered resulted in fatality for both mother and fetus.

HOW TO IMPROVE OUR UNDERSTANDING OF THE IMPACT OF TICK-BORNE INFECTIONS IN PREGNANT WOMEN AND THEIR INFANTS

From our review of the current literature on tickborne infections in pregnancy, we have identified a paucity of well-designed prospective studies and little investment in the accurate surveillance and monitoring of these infections worldwide. An independent group called the "ad hoc Committee for Health Equality in ICD11 Borreliosis Codes" was established in December 2015 to update the ICD11 codes for borreliosis diseases. One of the requests from this ad hoc Group was to have Congenital Lyme Disease instated as a "stand-alone" code and indeed in June of 2018 the WHO provided a provision code 1C1G.2 for Congenital Borreliosis. Having such a code would assist in the ability of researchers and advocates to petition for better studies and better funding to develop prospective studies and monitoring of women in pregnancy at risk and infected with tickborne infections; and longitudinally following up affected and infected children born to these mothers.

1C1G.2 Congenital *Lyme borreliosis* was removed in a very non-transparent manner from the ICD11 on December 17, 2018. Correspondence from a member of the ICD11 Medical and Scientific Advisory Committee of the WHO (MSAC) to an ad hoc member stated, *"This was in response to a request for the removal of Congenital Lyme borreliosis by the Public Health Agency of Canada (PHAC)..."*. Further communication from the WHO stated that there was no need for a "stand-alone" ICD code for Congenital Lyme as there was no recognized "congenital syndrome." However, such an argument is lacking in scientific credence, and congenital malaria has a stand-alone ICD code, despite not having a recognized "congenital syndrome." Despite multiple petitions and communications from members of the ad hoc Group and European MEPs and MPs to get clarification on the reason for "non transparent" deletion of the code from the proposed ICD 11, no response has been received from the responsible members of the WHO.

WHAT ARE THE CONSEQUENCES OF "MISSED" LYME DIAGNOSES TO THE UNBORN CHILD?

One unknown but plausible explanation for Autism Spectrum Disorders (ASD) is the possibility of a vertically transmitted infection in pregnancy. Bransfield et al. raise this issue and identify 24 infections and co-infections which may be contributing factors in the development of ASD in early childhood (42). Should this be the case he poses many unanswered questions; is the main mode of acquisition primary infection in infancy or from vertical transmission? Is the etiology of the disease caused by direct infection of nervous tissue or from a secondary immune response to infection, or both?

The evidence for a link between Lyme/tick-borne diseases (LY/TBD) and neuropsychiatric diseases in childhood has been raised from studies from the USA. Of the twenty states that reported the highest occurrence of Autistic Disorder per 10,000 people; fifteen reported a higher than average number of Lyme disease cases. Conversely, of the twenty states that reported the lowest incidence of Autistic Disorder per 10,000 people; zero reported a higher than average number of Lyme disease cases (43).

Although clinicians have previously suggested an association between Lyme disease and ASD, the first study provided a comprehensive case history review on the charts of 102 gestational LYD/TBD cases, and revealed that 9% had been diagnosed with autism and most were diagnosed with a broad spectrum of developmental disabilities. As a control, 66 mothers with Lyme disease who were treated with antibiotics prior to conception and during pregnancy; all gave birth to normal healthy infants (44) When children suspected with ASD were tested for Lyme, most studies demonstrate about 25% of ASD are infected with *Borrelia burgdorferi* (45).

It has been observed by Jones et al. that treatment of LYD/TBD during pregnancy can prevent the development of autism and other developmental disabilities associated with LYD/TBD (44). Another study has objectively demonstrated that antibiotic treatment can reduce ASD symptoms associated with LYD/TBD (43).

Another "mystery" childhood illness that has been attributed to congenital Lyme infection is Spinal Muscular Atrophy. Recent studies have suggested a connection between ALS and SMA (46).

SUMMARY

Tick borne infections are impacting on maternal and child health worldwide. The extent of this problem appears to be greater than current "status quo" acknowledges. Accurate data on the extent of tickborne infections worldwide is limited, and networks to monitor mothers and children following suspected tickborne exposure in pregnancy is essentially non-existent. Peer reviewed published articles on this disease area consists largely of case reports and small studies without adequate control. Many studies are retrospective in nature, which limits conclusions. However, these reports, limited in "quality" as they are, should represent a "red flag" to clinicians and public health officials within the health care system. And should be embraced, not ignored or discounted. The failure of recognition of Congenital Lyme both by clinicians caring for their patients, and by the WHO, who have failed to engage with the ICD 11 codes for Congenital Lyme, is a lost opportunity for better science and improved understanding. Such investment could result in improved maternal and child health, a clear purported declaration of the WHO, "no child left behind." Science needs to prevail, and politics rather than science have to date won the day. And the children are losing.

AUTHOR CONTRIBUTIONS

JL: literature review, writing of the manuscript, and opinions expressed are those of the author.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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Potential Patient-Reported Toxicities With Disulfiram Treatment in Late Disseminated Lyme Disease

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Recently, disulfiram has been proposed as a promising treatment for people suffering from persistent symptoms of Lyme Disease. Disulfiram has several distinct molecular targets. The most well-known is alcohol dehydrogenase, a key enzyme for detoxifying the organism after alcohol ingestion. Other targets and modes of action of disulfiram, that may present problematic side effects, are less commonly mentioned. The *French Federation against Tick Borne Diseases* (French acronym, FFMVT), which associates three main Lyme patient organizations, MDs and PhDs, has recently been alerted to severe and persistent toxic events in a patient suffering from a late disseminated form of Lyme Disease following disulfiram intake. FFMVT reacted by launching a national call to examine whether other patients in France following a similar treatment could be identified, and what benefits, or side effects could be reported. The statements of 16 patients taking disulfiram have been collected and are presented here. Thirteen out of 16 patients reported toxic events, and seven out of 16 reported benefits for at least part of their symptoms. Based on the collected observations, it seems too early to promote disulfiram as a promising new treatment until the reasons underlying the reported toxicities have been explored, and the results of a well-conducted double blind clinical trial published. The importance of taking into account patient-reported outcomes in Lyme Disease is underlined by the present study.

Keywords: lyme disease, disulfiram, benefit, patient-reported adverse drug reactions, risk

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INTRODUCTION

Each year, in the USA, about one person out of 1,000 develops Lyme Disease as declared by the general practitioners, which leads to a total of around 300,000 annual cases (1). Similar frequencies have been reported in Europe, in particular in France (2) and in Germany (3). The patients usually take antibiotics for a few weeks, and in most of cases they recover. However, after several months or even years, a fraction of these properly treated patients, will develop a post-treatment Lyme Disease syndrome (PTLDS) linked to pathogens injected by the tick, usually *Borrelia* bacteria, often associated with other bacteria like *Bartonella*, parasites like *Babesia,* or even viruses. Another group of patients develops a late disseminated form of the disease without having noticed any initial event, like the pathognomonic cutaneous *erythema migrans*, rendering more difficult the Lyme Disease diagnosis.

There is no consensus for the optimal treatment of these late forms of disease. The major difficulties in their diagnosis and in their treatment are reflected in the number of different names

they have been given: late Lyme Disease, persistent Lyme Disease, chronic Lyme Disease, PTLDS, and in France SPPT for "*sémiologie persistante polymorphe après morsure de tique*" according to the new French government guidelines.

In such a context, there is a desperate need for many people to receive the optimal treatment. Recently, a new treatment has been reported, and many patients are currently trying it despite the fact that the main active compound, disulfiram (DSF), has never been clinically evaluated in the context of a chronic infection, either alone or in combination with antibiotics. In the present work, to try to answer this pressing issue, we have analyzed the scientific literature on DSF and collected patient-reported results in order to inform patients suffering from late forms of Lyme Disease of the potential risks or benefits of DSF treatment.

METHODS

After an alert in October 2019 from a patient presenting severe and persistent symptoms after taking DSF, the FFMVT (*French Federation against Tick Borne Diseases*) decided to launch two actions. One was a thorough analysis of the scientific literature, in order to try to understand the possible causes of such an apparent toxicity. The other one was to collect reported-outcomes from Lyme Disease patients having taken DSF. Three associations of patients, *France Lyme, Lympact, Relais de Lyme*, sent a standardized questionnaire, prepared by the authors, to their members suffering from PTLDS as described, among others, by J. Aucott (4), or from SPPT, the term used in France by *Haute Autorité de Santé* (High Health Authority) (https://www.has-sante.fr/portail/jcms/c_2857558/fr/borreliose-de-lyme-et-autres-maladies-vectorielles-a-tiques) and in a case law of the French Council of State (https://www.conseil-etat.fr/fr/arianeweb/CE/decision/2019-12-04/423060).

Concerned patients who were willing to contribute to this enquiry sent back the appropriate information on their clinical status and disease. Information requested included age, sex, health state, dosage and duration of the DSF treatment, concomitant medications, self-reported health improvements and potential toxicities. The answers were collected over a 2-weeks period, and anonymously transferred from patient associations to the authors of the present paper, before being tabulated and analyzed, as presented in **Table 1**. Note that the Research Integrity Specialist of Frontiers asked us to omit the gender information, and not to indicate the precise age, to reduce the risk for the patients to be indirectly identifiable.

This enquiry allowed us to rapidly collect the appropriate information for evaluating whether or not reported severe adverse events in a first patient were exceptional or not. However, no definite conclusion can be drawn under such conditions, taking into account the sample size, the different doses of DSF used, and different combinations of concomitant medications used.

RESULTS

The first part of this section will present potential reasons why toxicity is expected in patients taking DSF, and not exclusively following alcohol ingestion. The second part will concern the analysis of 16 patient-reported outcomes collected in November 2019 in France.

DSF, an ALDH Inhibitor

DSF has been clinically used for nearly 70 years, essentially for treating alcohol dependence. DSF inhibits an enzyme that is required for full alcohol degradation, preventing the detoxification that should follow alcohol drinking. This leads to severe nausea and discomfort in DSF-treated patients when they drink alcohol. This induced association between alcohol and severe discomfort is the basis of DSF use for the treatment of alcohol-dependent patients. More than 3,000 scientific publications mention DSF in their title, and most of them are related to alcohol consumption.

After ingestion, alcohol (ethanol) is degraded in two steps:

$$\text{Ethanol (CH3.CH2.OH)} \xrightarrow{ADH} \text{Acetaldehyde (CH3.CHO)} \xrightarrow{ALDH} \text{Acetate (CH3.COO}-\text{)}$$

The first reaction is catalyzed by alcohol dehydrogenase (ADH), the second one by aldehyde dehydrogenase (ALDH). The final product, acetate, has no toxicity. By contrast, acetaldehyde (AcH), also known as ethanal, is much more toxic than ethanol. Ethanal is quite volatile, and at low concentration gives off a pleasant smell of green apple, whereas at higher concentrations, its smell becomes pungent. Acute AcH toxicity may involve in particular the nervous system (5). In long term exposure, AcH is also a carcinogen (6). Note that ALDH is only weakly expressed in 30–40% of Asian, individuals, preventing them from properly eliminating alcohol, which explains why many of them have a low tolerance to alcohol.

The potent DSF-induced ALDH inhibition is copper-dependent (7). *In vivo*, DSF is cleaved, giving rise to diethyldithiocarbamate, an efficient copper chelator (8). Through this mechanism, DSF inhibits copper-dependent enzymes, such as ALDH, abundant in the liver (9), or dopamine β-hydroxylase in the brain (10). The best described effect of DSF, but not the only one, is its toxicity in the presence of alcohol, and sometimes even in its absence, as discussed below.

There are two main places in the organism where the enzymes ADH and ALDH allow the degradation of alcohol to acetate. The first is the ALDH-rich liver, which plays a key role after alcohol drinking. The second, which is seldom mentioned but nevertheless quite important, is the microbiota of the digestive tract, with its billions of bacteria and fungi particularly abundant in the mouth and the large intestine. In some bacteria, the ADH enzymatic activity is significantly stronger than the ALDH one. As a result, in the presence of alcohol, such bacteria, including the commensal ones, trigger an increase in the concentration of toxic AcH (11). This might contribute to a higher frequency of mouth and throat cancers in alcohol-dependent patients (12).

In addition, some anaerobic bacteria and yeasts are able to convert glucose into ethanol (this "alcoholic fermentation" is the basis for the manufacturing of alcoholic beverages). Under certain culture conditions, it is possible, when supplying some of these microorganisms only with glucose, to generate alcohol

TABLE 1 | Responses of 16 patients to DSF treatment (France, July–November 2019).

Patient	Age	Subtype of disease	DSF treatment (in mg/day, duration, sequence)	Other treatment	DSF attributed benefit (patient self-report)	DSF attributed side-effects (patient self-report)
#1	21–25	Late L.D.	250 (1 d/3 for 1 w), (1 d/2 for 1 w), (1 w), 500 (10 d), 250 (3 d)		No	Cranial neuropathy, very strong headaches, anxiety, suicidal thoughts, loss of sleep, hot flushes, disturbed intestinal transit, osteo-articular pain, tremors, tinnitus linked to head movements, hyperacusis. Stopped the treatment, but major side effects persisted after 1 month.
#2	41–45	Late L.D.	250 (5 w), 125 (8 w)	Antibiotic	No	Strong dizziness and nause.
#3	66–70	Late L.D., associated with a suspected lupus	500 (4 w)	Hydroxychloroquine	Clear decrease of fatigue and pain	No
#4	46–50	Late L.D.	250 (2 w), 500 (7 w), 750 (2 w)		Too early	Fatigue, general pains, loss of sleep, tachycardia, paranoid delirium requiring stopping the treatment.
#5	36–40	Late L.D.	62.5 (1 d/3 for 2 w), 62.5 (2 d/3 for 1 w), currently 62.5 (1 w)	Antibiotic, hydroxychloroquine	No	No
#6	46–50	Late L.D.	250 (3 w), a 24 h break, 62.5 (3 w)	Nitazoxa–nide	After a 24 h break, a major effect on fatigue and pain	At 250 mg per day, strong Jarish-Herheimer reactions, with headaches, cranial neuropathy, fatigue, pain.
#7	31–35	Late L.D.	500 (4 w)	Antibiotic	No	No
#8	61–65	Late L.D.	250 (4 w)		No	Important nausea, anorexia, significant weight loss, exacerbation of pain.
#9	45–50	Late L.D.	250 (6 w), 500 (1 w) at night		A decrease of pains was observed	Significant concentration problems when taken during the day. Causes significant disturbances of immediate memory, speech difficulties, headaches and dizziness.
#10	46–50	Late L.D.	125 (2 w)	Antibiotic	No	Flu-like symptoms, increased pain in some joints.
#11	46–50	Late L.D.	250 (4 w)	Antibiotic	Improvement of tone and general health	Dizziness and nausea.
#12	56–60	Late L.D.	250 (1 d/3 for 1 w), 250 (1 d/2 for 1 w), 250 (1 w), 500 (1 w), a 2 w stop		Few days after ending treatment, a real positive effect on the general health with a decline of the majority of symptoms was observed, but 8–10-day later some of them reappeared (leg weakness, joint pain)	Increased pains in the whole body, especially in joints. Important fatigue, flu-like symptoms, low blood tension, eye irritations, abdominal pain, mild nausea.
#13	76–80	Late L.D.	250 (4 w)	Antibiotic	No	Strong dizziness, leading the patient to stop treatment at day 3.
#14	51–55	Late L.D.	From 125 to 500 over 4 w, a 4 w stop, 500 (4 w), 2 w stop, and currently 500		Better sleep. Slight decrease in peripheral nervous system pain (main symptom of this patient)	Fatigue, drowsiness, hot flushes, disturbed intestinal transit.
#15	51–55	Late L.D.	500? (4 w)		No	Major concentration difficulties, strong dizzines, and strong headaches. Important fatigue requiring to lie down.
#16	31–35	Late L.D.	250 (currently under treatment since 3 w)		Improved concentration et cognition. And decrease in headaches and fatigue	Two important Jarish-Herxheimer reactions.

DSF, Disulfiram; L.D., Lyme Disease; w, week; d, day.

and then AcH. Thus, the yeast *Candida albicans* is capable of producing high levels of toxic AcH, after glucose fermentation (11). It can thus be predicted that the toxicity of DSF should be particularly marked in people suffering from candidiasis.

Finally, other bacterial families, such as *Lactobacillus*, have an ALDH activity larger than that of ADH, which makes them good detoxifiers, by preventing the accumulation of AcH (6).

Other Modes of Action of DSF

Although the DSF toxic effects occurring in the treatment of alcohol-dependent patients have been known for a long time, additional effects have been described more recently. It has been shown in particular that, *in vitro,* DSF can be cytotoxic for cancer cells (13). These results prompted the launch of three clinical trials including DSF in the treatment in prostate, pancreas and glioblastoma cancers (https://www.cancer.gov/about-cancer/treatment/clinical-trials/intervention/disulfiram). None of these trials, started in 2016 and 2017, has yet given rise to publication.

It was initially thought that these newly discovered effects were also due to the inhibition of ALDH, but this is not always true, and several other DSF targets have been identified. Thus, the protein NLP4, which is necessary for the cellular response to various stresses, is inhibited by DSF-copper complexes (13, 14). In addition, DSF can block an intracellular detoxifying pathway. It can inhibit the proteasome (15), a multi-protein complex required for the elimination of improperly folded proteins. DSF can also block the activation of NF-κB (15, 16), a key molecule in inflammatory stresses, known for inhibiting apoptosis.

In vitro, DSF can neutralize a DNA methyltransferase involved in DNA repair (17). It may also inhibit P-gp, a multidrug pump responsible for the extrusion of toxic molecules, which contributes to cellular resistance to many cytotoxic molecules (18). The effect of DSF on NF-kB, DNA repair, and P-gp may all contribute to the *in vitro* effects of DSF against tumor cell lines. The effect of DSF on P-gp has been more particularly studied in fungi and yeasts, offering a possible explanation for the antifungal effect of DSF (19). However, some authors have attributed DSF anti-fungal properties to its capacity to elicit oxidative stress in yeasts (20). Still *in vitro*, DSF also displayed toxic effect against *Plasmodium falciparum*, the causative agent of malaria (21), and also against some bacteria (22).

As mentioned previously, DSF can act as a copper chelator, thus inhibiting copper-dependent enzymes. Some bacteria express such enzymes, rendering them sensitive to DSF. However, it is unclear whether the anti-bacterial effect of DSF is due to copper depletion or to direct effects of copper complexation inside bacteria (8, 23).

Most demonstrations of an antibacterial effect of DSF were performed *in vitro* (24, 25), at concentrations not always compatible with its *in vivo* use. For instance, one study claims that DSF is toxic to *Mycobacterium tuberculosis*, including the dormant form, both *in vitro* and *in vivo* (24). In fact, the experimental protocol allowed the evaluation of the effect of DSF on the global bacterial load, but showed nothing on *in vivo* bacterial dormancy. In this study, the efficient dose of DSF would have been equivalent to 1,100 mg of DSF / day for a human of 70 kg, well above the dose tolerated by Lyme Disease patients (see below). Thus, the conclusions of this study still remain to be validated.

In summary, DSF is a pleiotropic drug with multiple targets, without specificity for one molecule or a single pathogen. Most of the reported anti-bacterial effects of DSF have been obtained *in vitro,* making it difficult to extrapolate for its *in vivo* use, especially when used in combination with antibiotics.

A Clinical Trial With DSF for Treating Lyme Disease

In March 2019, Pr. Brian Fallon started a clinical trial using DSF and including 24 Lyme Disease patients (https://clinicaltrials.gov/ct2/show/NCT03891667). The results of Professor Fallon's study should provide important information in the near future. On the clinicaltrials.gov website, the Study Description indicates that DSF is active against Borrelia's dormant form. However, evidence to support this claim is not provided. The clinical trial document refers to three previous articles (22, 26, 27). In 2016, Pothineni et al. published an *in vitro* high-throughput screening of more than 4,300 drug candidates, against *Borrelia burgdorferi* grown to its stationary-phase (26). DSF appeared to be a very efficient bactericidal molecule for *Borrelia in vitro*, but no *in vivo* results have been reported yet. In 2017, Dr. Long has shown that, *in vitro*, DSF is cytostatic for Gram-positive bacteria, such as *Staphylococcus* or *Streptococcus*, but not for Gram-negative species (22). Finally, in 2019, Dr. Liegner reported three cases of patients who had been treated with DSF after a Lyme Disease that had lasted for several years with heavy treatments (27). For instance, at one point, one patient simultaneously took amoxicillin, clarithromycin, hydroxychloroquine, metronidazole, atovaquone / proguanil, and amitriptyline. After 9 years of illness he took DSF for 3 months: the symptoms of the Lyme Disease seem to have disappeared but the patient had a temporary psychiatric hospitalization. The second patient was on DSF for 6 weeks. The symptoms of the Lyme Disease improved but the treatment was stopped following a syncope, which resulted in a concussion and required hospitalization. In summary, the Liegner study reports three cases in which DSF seems to have been effective against late Lyme Disease, but in two of them neurological problems occurred during the treatment. These three cases have attracted considerable attention and raised great hopes in the Lyme Disease patient communities. However, in a recent talk at the 2019 ILADS Symposium, Dr. Liegner presented data on 30 Lyme Disease patients that he had treated with DSF. In 18 of them, DSF provoked either peripheral neuropathies or psychiatric problems, or both.

DSF Neuronal Toxicity ?

For tens of years, it has been known that DSF can cause occasional and sometimes severe neuropathies (28). In optic neuropathies, with a partial loss of vision, recovery took about 6 months after stopping DSF (29). When DSF is used to treat alcohol dependence, the incidence of undesirable neuropathies has been estimated as 1/15,000 (30). As for the totality of the undesirable effects caused by the DSF, their frequency has been evaluated at 1 per 200–2,000 patients (9).

Are DSF associated neurological disorders (neuropathies or psychiatric problems) related to DSF anti-ALDH activity leading to AcH synthesis? It has been demonstrated that, *in vitro*, AcH can have an acute toxicity on neurons due to an increase of reactive oxygen species, but this observation has not been extended *in vivo* (5).

An AcH increased toxicity could theoretically occur even in the absence of alcohol intake, for example in patients with *Candida* infections, or harboring a high load of microorganisms capable of alcoholic fermentation. Alcoholic fermentation, typically performed by yeasts, should be distinguished from lactic fermentation, more common in anaerobic bacteria. A few cases have been reported of people suffering from Gut Fermentation Syndrome (31, 32). Such patients had up to 2 g/L of alcohol in their blood, without any alcohol intake. This alcohol was produced by fermentation by large colonies of the yeast *Saccharomyces cerevisiae* in their intestine.

Another DSF target is dopamine β-hydroxylase, a copper-dependent enzyme, responsible for converting dopamine (DA) to norepinephrine (NE) in noradrenergic neurons. This enzyme is mostly expressed in the brain, adrenal gland and liver (https://www.proteinatlas.org/ENSG00000123454-DBH/tissue). By inhibiting dopamine β-hydroxylase, DSF simultaneously reduces NE and elevates DA in these tissues. A link has been established between psychosis and DSF-induced increase of DA in the mesolimbic system (10, 33). Dopamine β-hydroxylase is also expressed in some peripheral sensory neurons and it has been suggested that neurotoxic products of catecholamines metabolism in nociceptors can cause neuronal dysfunction underlying neuropathic pain (34).

Patient-Reported Outcomes

We have recently received from French associations of Lyme patients the results of an enquiry sent to their members suffering from persistent Lyme Disease. The main questions were: have you taken DSF as a treatment for your disease? Which benefits or side effects did you experience? 16 patients have answered.

The clinical features most frequently reported were major fatigue, articular pain and cognition complaints mainly involving memory, whether or not patients were seropositive for *Borrelia*. The results are presented in **Table 1**. The conclusions are: 13 out of 16 patients experienced DSF-induced toxic or side effects, mainly concerning the nervous system (neuropathies, headaches, dizziness, difficulty of concentration and expression, sleep disturbance, general pain increase, increase in general fatigue). Several patients reported a more specific increase in their osteo-articular pains, nausea or intestinal disorders.

When taking DSF, some patients simultaneously experienced both negative effects on some symptoms and improvement of others. All in all, 7 out of 16 patients perceived benefits mainly on fatigue and pain, especially after stopping DSF. Others could not differentiate whether partial improvements were due to DSF or to the antibiotics taken during the same period.

Some of DSF toxic effects observed in Lyme patients could be due in part to high initial DSF doses, similar to those used for alcohol-dependent patients. On the other hand, some of these effects could have been due to Jarisch Herxheimer reactions triggered by DSF-induced death of *Borrelia*. However, some patients, who had already experienced Jarisch Herxheimer reactions before, reported that some of the reactions encountered with DSF treatment were clearly of a different nature. Collectively, these observations suggest that patients with persistent Lyme Disease are more sensitive to the toxicity of DSF than people who have been treated for alcohol dependence, and that in these patients, DSF-induced toxicities are not all related to Jarish Herxeimer reactions.

DISCUSSION

Published scientific articles allow us to draw the conclusion that, *in vitro*, DSF can undoubtedly kill certain bacteria strains, and that *in vivo*, DSF can be toxic to both bacteria and the human body. These toxicities can be both acute and long-term.

One can propose different hypotheses to explain these toxicities. They might be mediated by the inhibition of copper-dependent enzymes, such as ALDH or dopamine β-hydroxylase, or the blocking of the NLP4 molecule, or through an oxidative molecule increase, and possibly through yet unidentified mechanisms. Part of these toxicities may also depend on the microbiota, in which some bacterial or yeasts species have a propensity to produce fermentation-derived toxic AcH. It would be worth testing if any intake of bacteria such as *Lactobacillus*, which have a high ALDH activity, could be used to counter the DSF-induced toxicities.

On the other hand, many studies have reported that patients with PTLDS have an increased sensitivity to pain, which can affect vision, hearing, touch, and even smell, as reviewed by Batheja et al. (35). These chronic pains can be related multiple to chemical sensitivity and chronic fatigue syndrome, in which the pain sensitivity is modified as well, as reported in Gulf war veterans (36). There is increasing evidence for abnormal sensory processing in these syndromes, with a low "unpleasantness threshold" for multiple types of sensory stimuli (37).

The differences observed for effective concentrations of DSF between alcohol-dependent patients and those suffering from PTDLS or SPPT could also be linked to a such central sensitization often observed in patients suffering from borreliosis (35).

It is necessary to understand why DSF toxicity appears particularly severe and frequent in patients with Lyme Disease, and to rapidly explore the reasons for such DSF toxicity in Lyme Disease animal models. Until we have the first answers to this question, it would be premature to consider DSF as the new miracle molecule for patients suffering from late disseminated Lyme Disease.

Basic Science vs. Social Networks

Case reports are a very useful approach for drawing attention to the possible effectiveness of a new treatment. Undoubtedly, the case report published by Liegner (27) has played such a role. However, the next logical step should have been to examine the potential toxicity of DSF for Lyme patients. This could have been achieved first by using animal models, and then within a standardized clinical trial. These steps were rapidly short-circuited, due to the strong social demand for Lyme Disease treatments. This pressure is exerted largely by social networks, emphasizing their speed and efficiency, but at the same time a lack of analysis and scientific rigor.

Importance of Patient-Reported Outcomes

Following the rapid spread of the idea that DSF could be a major improvement for the treatment of late Lyme Disease, hundreds of patients began using DSF in the hope of treating their disease. At this point, it is important to require, as we do here, on rapid feedback from the patients themselves. No one knows better than patients the severity and importance of secondary toxicities from treatment. They know themselves better than physicians, who sometimes tend to overestimate the benefit/risk ratios (38, 39).

The limitation of the present study is linked to the small number of included patients. This highlights the need for follow-up studies with a larger number of patients to specify the risk/benefit of DSF in late Lyme Disease. The results and experiences reported by the patients should be included in these studies to determine how many of them have truly benefited from DSF treatment. Aiming at distinguishing Jarish Herxheimer reactions due to bacterial die-off and toxic side effects of the drug will be an important issue. More generally, a patient survey will have to be designed to evaluate how many patients have benefited of DSF and how many have not. A long term follow up of the DSF treated patients using an online patient feedback tool will be necessary to determine if they have any relapse or stable remission. All this information is necessary to determine the risk/benefit ratio of DSF for Lyme Disease. This will require a close collaboration between patients, doctors and researchers.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

An ethical review process was not required according to national legislation and institutional requirements for the present study. Patient treatment was decided by their physician independently of the study. Patients associations were asked by the authors to collect patient-reported outcomes, which have been transferred anonymously to the authors. All patients that participated to this enquiry were volunteers and have given a written agreement to participate in this study.

21 patients had initially sent a patient-reported outcome. Following your recommendation, they were all asked to give their agreement to participate to this study. No one refused, but five of them did not answer. Therefore, the revised manuscript only presents the 16 cases of patients who sent their written consent.

AUTHOR'S NOTE

AT and HG are members of the scientific council of *Fédération Française contre les Maladies Vectorielles à Tiques* (FFMVT), and RG is president of FFMVT.

AUTHOR CONTRIBUTIONS

AT: analysis of the literature. HG, RG, and AT: synthesis and analysis of the patient-reported outcomes. RG: medical advice. AT and HG: writing the manuscript.

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REVIEW
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A Practical Approach to the Diagnosis of Lyme Borreliosis: From Clinical Heterogeneity to Laboratory Methods

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Clinical evaluation of Lyme Borreliosis (LB) is the starting point for its diagnosis. The patient's medical history and clinical symptoms are fundamental for disease recognition. The heterogeneity in clinical manifestations of LB can be related to different causes, including the different strains of Borrelia, possible co-infection with other tick transmitted pathogens, and its interactions with the human host. This review aims at describing the heterogeneous symptoms of Lyme Borreliosis, as well as offering a practical approach for recognition of the disease, both in terms of clinical features and diagnostic/research tools.

Keywords: Lyme disease, Borrelia, clinical symptoms, diagnosis, clinical heterogeneity

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INTRODUCTION

The genus Borrelia includes three Groups: Lyme Borreliosis (LB), Reptil Associated (REP), and Relapsing Fever (RF) Group (1).

Lyme disease or Lyme borreliosis (LB) is an anthropozoonosis, caused by different genospecies of the *Borrelia burgdorferi sensu lato* complex. The main tick vector for Borrelia species in Europe is the *Ixodes ricinus* (2), in America the *Ixodes scapularis* and *Ixodes pacificus* (3–5), while in Asia (6) and Russia (7) it is the *Ixodes persulcatus*. These ticks are possible vectors of Lyme Borreliosis (LB) as well as other pathogens, including viruses, intracellular bacteria, and Protozoa which can co-infect humans (LB co-infections) (8, 9). There are several *B. burgdorferi sensu lato* genospecies, directly associated with human LB. However, only three genospecies, namely *Borrelia burgdorferi sensu stricto*, *B. afzelii*, and *B. garinii*, have been systemically related to LB (4, 10). In addition, four other genospecies have been occasionally detected in humans: *B. bissettiae* (4, 5), *B. lusitaniae* (6, 7), *B. spielmanii* (8), and *B. valaisiana* (9), especially in Europe (11). Specificity in terms of dominating hosts has been reported both across and within continents (12, 13). The spatial distribution of the different genospecies allocates *Borrelia burgdorferi sensu stricto* in North America [and possibly *B. mayonii*, although this causes a disease somewhat distinct from typical LB (14)] and five species in Europe and Asia, *B. afzelii*, *B. garinii*, *B. burgdorferi*, *B. spielmanii*, and *B. bavariensis* (15). The heterogeneity in terms of genospecies can mirror different clinical manifestations of LB due to host specialization and tissue tropism. Although overlapping, distinct spectra of clinical manifestations have been recognized for the three main genospecies. In detail, *B. burgdorferi sensu stricto* is mostly associated with arthritis and neuroborreliosis, *B. garinii* with neuroborreliosis, and *B. afzelii* with chronic skin conditions such as acrodermatitis chronica atrophicans (10).

Spirochetes circulate in small amounts in the blood even in acute LB patients (16), with the exception of *Borrelia mayonii* which has been reported to cause high spirochetemia (14, 17). Depending on the case and genospecies, they can grow in several tissues (18), including skin, nervous and joint system, although less frequently LB can also affect eyes, heart, spleen, and other tissues.

Based on the spatial variability of Borrelia, for an accurate diagnosis, it could be useful to know if the patient has visited other countries or continents.

Some clinical aspects that can be helpful for a correct diagnosis of LB will be described hereafter. **Figure 1**, instead, shows an overview of possible overlapping scenarios defining LB. Furthermore, a brief description of laboratory investigation tools is included at the end of the review.

TICK-BITE WITHOUT ERYTHEMA MIGRANS

Patients sometimes seek medical assistance after a tick bite. In this case, the first step is to remove the tick with small tweezers or an *ad hoc* tool at the level of the rostrum. Afterwards, it is important to inform the patient of the symptoms, which, in the case of Borrelia infection, may develop in days/weeks. It is also possible to submit the tick for identification and testing for different pathogens. The identification of pathogens within the tick defines a possibility, not the certainty of developing LB (19).

ERYTHEMA MIGRANS (EM)

Recognition of an EM rash is very important in LB as it is a hallmark symptom of LB, even when the patient does not recall the tick bite. However, as it has been observed, in rare cases the tick can still be attached to the center of the EM (20, 21). The geographical area where the patient was bitten as well as the date are important elements that should be gathered from the patient. Other variables to establish are: the time elapsed between the tick bite and the appearance of the erythema (usually 5–30 days) and its diameter, especially if larger than 5 cm (22). The most important diagnostic criterion is the EM centrifugal evolution. Erythema migrans (**Figure 2**) is pathognomonic for LB, therefore it should be treated immediately as serology testing to confirm infection is not necessary. Nevertheless, the clinical presentation of an EM can vary considerably (23). Several clinical variations have been observed, such as smaller-sized-EM of about the size of a coin, oval shaped EM with no darker outline, red-violet

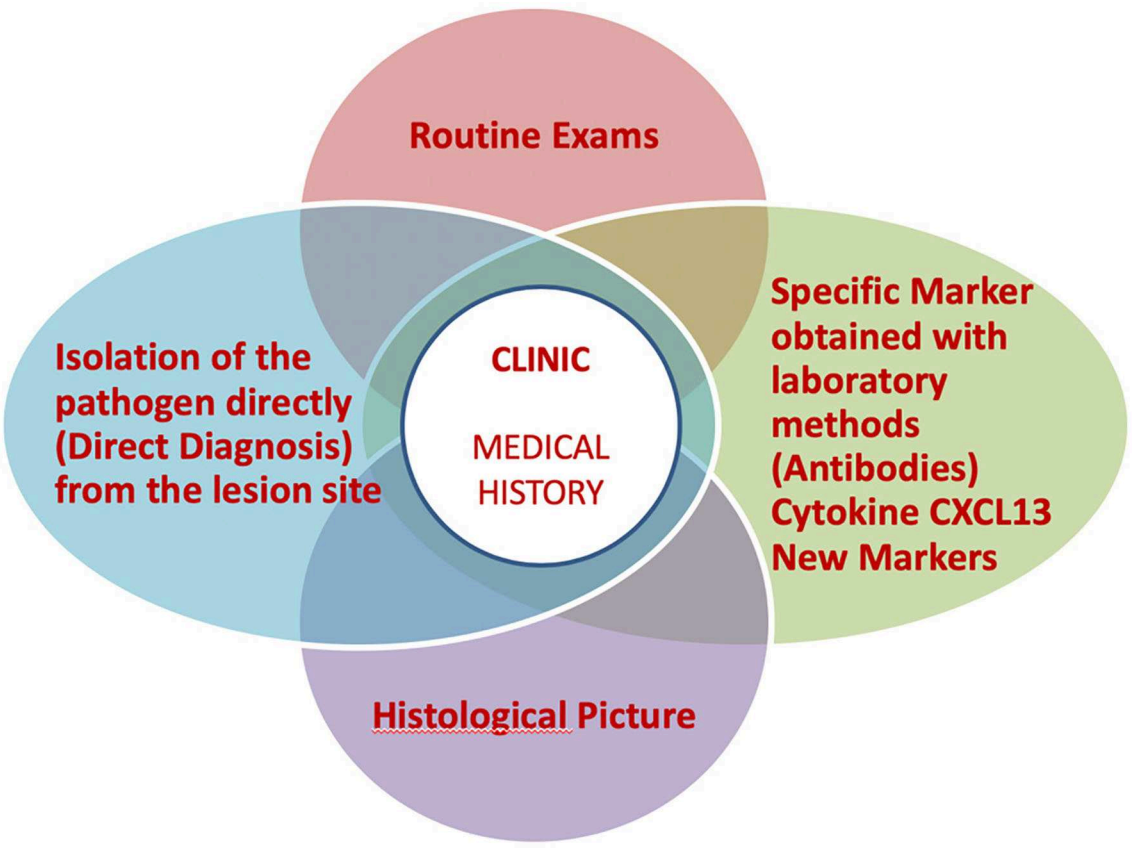

FIGURE 1 | Overview of LB management.

EM (erysipeloid), EM with vesicles which mimics herpes simplex or herpes zoster (24), painful EM (burning), itchy EM, hidden EM (scalp), and EM with atrophic evolution (25). It has been shown that in some cases of EM, Borrelia infection can already be disseminated (26).

Differential diagnoses include: mycosis fungoides, granuloma annulare, and interstitial granulomatous dermatitis (IGD), tinea corporis (mini EM), and erythema necroticans migrans.

Serological testing is not recommended because of their poor sensitivity in the early stages of LB. In order to achieve the best outcome for patients, antibiotic treatment should be started without delay.

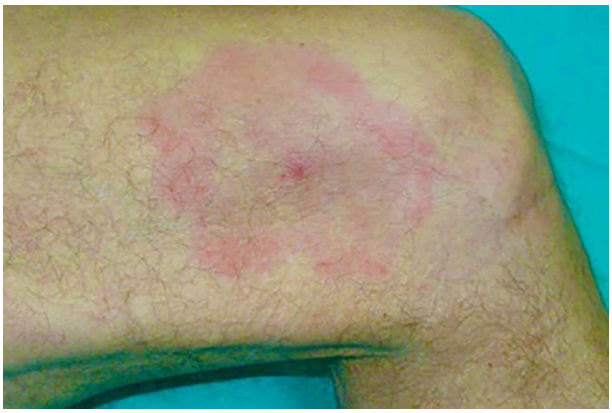

FIGURE 2 | Erythema Migrans of the thigh.

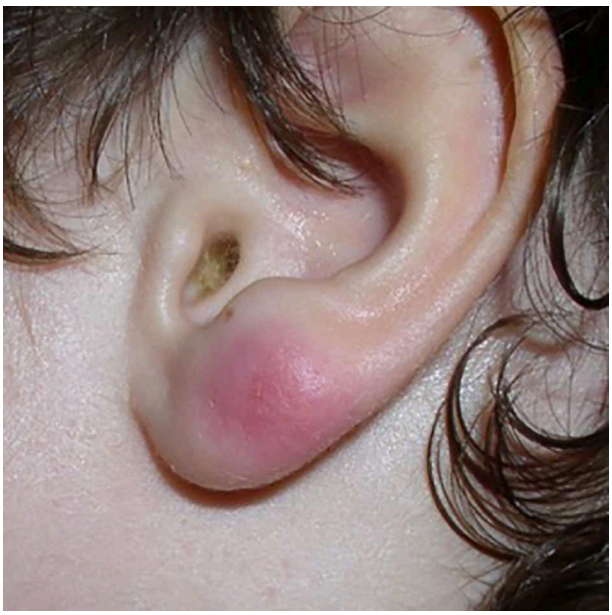

FIGURE 3 | Borrelia Lymphocytoma of the ear lobe.

CUTANEOUS MANIFESTATIONS EXCLUDING THE ERYTHEMA MIGRANS

Multiple Annular Erythema

Secondary EM is characterized by multiple erythematous lesions, which do not develop round the site of the tick bite. It can consist of a few or several plaques that can be located throughout the body (27). The lesions are multiple and can vary from a few cm to more than 20 cm, and are more frequently observed in children (22). The presence of multiple annular erythemas may precede the onset of neurological manifestations, especially in adults.

Borrelia Lymphocytoma

Borrelia lymphocytoma is defined as a B-cell pseudo-lymphoma that occurs in response to the presence of Borrelia antigens in the skin. Borrelial lymphocytoma can develop when EM is present and mimics a tick-bite reactive nodule. It is relatively frequent in Europe, while it is seldom observed in the US, because in most cases it is caused by *Borrelia afzelii* and more rarely by *B. garinii* and *B. bissettii* (28). Clinically, it appears as a solitary (rarely multiple) soft and non-tender bluish-red nodule or plaque with a size between 1 and 5 cm, sharply demarcated. It is typically found on the ear lobe (**Figure 3**), the mammary areola, and less frequently on the scrotum or the axillary fold. Extra-cutaneous signs and symptoms are very infrequent. The presence of Borrelia biofilm in human infected skin tissues has been demonstrated (29).

In the presence of this clinical manifestation the following exams should be performed: serology for *Borrelia burgdorferi* (ELISA and Western-Blot), β2-Microglobulin, and serological tests for Ehrlichia (Anaplasma) (30). Histological examination of skin biopsy and immunohistochemistry to define immunophenotype are also suggested (usually CD20 positive, Bcl-2 negative, κ and λ light chain expressed in an equivalent manner and Borrelia-PCR on DNA from skin slides).

Differential diagnosis includes cutaneous marginal zone lymphoma (PCMZL, **Figure 4**), which clinically and histologically may present similarities at the immunophenotype. PCMZL is generally CD20, CD22, CD79a, and BCL-2 positive, whereas it is CD5, CD10, Bcl-6, and CD23 negative, and the κ/λ light chain ratio in the histological tissue is very high (31). Borrelia's detection in PCMZL is included in the EORTC guidelines (32, 33).

PCR for Borrelia on tissue's DNA (frozen or formalin-fixed and paraffin-embedded) can target OspA as reported by Cerroni (34), but also p41 (flagellin) and p66 (35). Skin biopsy specimens from the site of the lesion can also be submitted for culture and isolation of Borrelia.

Acrodermatitis Chronica Atrophicans (ACA)

ACA is the pathognomonic symptom of late LB. Patients, at presentation, should be asked whether they remember being bitten by a tick several months or even years before and whether they ever had an EM. Since the clinical appearance of ACA is not distinctive, it is of key importance to be generally alerted of the possibility of ACA in patients

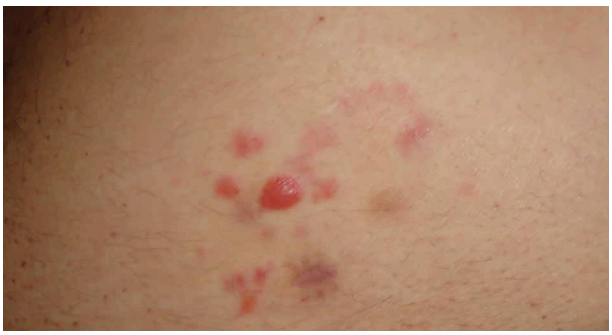

FIGURE 4 | Primary cutaneous B cell marginal zone lymphoma of the trunk. Of note the image that has been already published refers to the same patient but it is slightly different from this one.

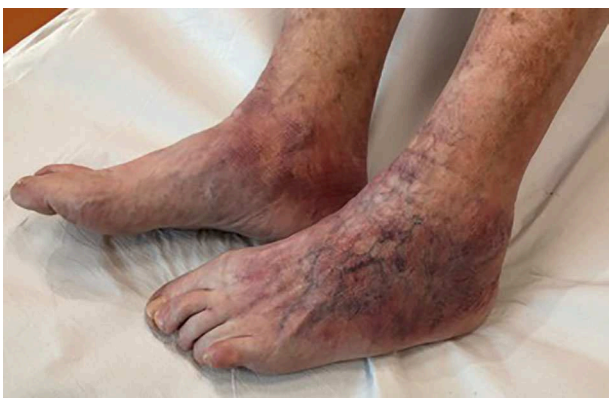

FIGURE 5 | Acrodermatitis chronica atroficans of the legs.

with bluish-red discoloration of a limb with or without swelling and/or atrophy, especially where LB is endemic (36, 37).

Unilateral acrocyanosis is present in the initial phases. This feature is followed by atrophy of the upper and/or lower limbs in an asymmetric manner, which, due to thinning and consequent greater transparency of the skin, allows the vessels of the dermis to be more visible. This condition leads over time to thinning of the most involved limb (22). ACA (**Figure 5**) is usually localized on the limbs, however, the face is also an acral site, and in some cases, it is difficult to distinguish the ACA of the face from Parry-Romberg syndrome, which may be a variant (38).

In addition to ACA, in some cases, other atrophic-sclerodermic manifestations may be related to LB (39, 40).

Serology by chemiluminescence is usually very high in VlsE IgG; in Western-Blot, p93 (p83/100) and DbpA are generally observed.

Skin biopsy for histological examination and PCR for Borrelia are also possible for research purposes. Isolation of Borrelia in BSK medium from skin lesion can result in the growth of *Borrelia afzelii* (or more rarely *valaisania*, *lusitaniae*, or *yangtze*).

TABLE 1 | Articular Involvement in LB.

Features	Location	Clinic
Mono/Oligo Involvement	Large Joints	Swelling
Asymmetry frequent attacks	Knee Joint most affected	Marked functional Impotence
		Skin Nodules
		Absence of Stiffness in the Morning

Other Possible Skin Manifestations

Other possible skin manifestations that have been associated with LB are: urticaria (41), purpura (42), and erythema nodosum (Baggio-Yoshinari syndrome) (43).

GENERAL AND EXTRACUTANEOUS CLINICAL MANIFESTATIONS

General Symptoms

Important information to be obtained from patients includes: the geographical area where the patient lives (if endemic or not for LB), if, in the previous weeks or months the patient has been in wooded areas, if he/she has traveled or has been camping, or has spent time in public parks and gardens or if he/she owns any pets. Requested information should also include the date of the onset of symptoms, recollection of a tick bite and/or of a circular erythema as well as the location and the duration of the skin lesion. In the case of a positive, response, the patient should be asked if he/she was previously treated with antibiotics, what type of antibiotics, and what the duration of treatment was. Other clinical manifestations can be fever, lymphadenopathy, balance disorders, dizziness, and photophobia (44).

Joint and/or Muscular Symptoms

Arthritis occurs after 4 days to 2 years (average, 6 months) from EM (45–49). In a European group of patients, the period between the tick bite or EM to the onset of arthritis ranged from 10 days to 16 months, with an average of 3 months (50). A summary of the articular involvement of LB is reported in **Table 1**.

In the early phase, the patient presents mono- or oligoarticular migrant arthralgia at the level of the large joints. The first affected joint is often near the site of the EM or the tick bite. However, sometimes other large or small joints, such as the temporomandibular joint (TMA), are also affected (51). Over time, the duration of joint arthralgia tends to lengthen, while painless intervals become shorter.

The articular involvement in the late phase has different clinical features compared to the typical migrant myo-arthralgia of early LB. The clinical symptomatology is not easy to distinguish from arthritis due to other causes. The disorder can become chronic or intermittent, with attacks lasting from a couple of weeks to a few months, which can be followed by resolution of symptoms. The intensity of the attacks decreases

over time. Hyperpyrexia is not usually present, but a general sense of fatigue is common.

Swelling of the joints with marked functional impotence is often present. Affected knees, for instance, may have very large effusions (synovial fluid) (52). If those injuries are not diagnosed and treated, the patient will possibly experience erosion of the cartilage and bone which can lead to permanent damage of the joint.

Muscular system involvement includes myalgia, muscle weakness, and myositis (53) with difficulty in raising the arms above the head, carrying weights, and climbing stairs; and dysphagia, with difficulty breathing due to the involvement of intercostal muscles (inter-costal diaphragm). In some cases, these symptoms can simulate a dermatomyositis (41).

To confirm diagnosis, it is useful to perform a serological ELISA test followed by a Western Blot. In case the patient reports having headaches and/or a fever, tests for TBE, Ehrlichia (Anaplasma), Rickettsia, and Bartonella coinfections are suggested. A rheumatologic examination can be also requested.

Serum IgG antibodies for *B. burgdorferi s.l.* are present in high titers in patients with Lyme arthritis, while a negative IgG serology rules out the diagnosis (54, 55). Serological investigation of synovial fluid is not helpful because of the absence of a blood–synovial barrier; IgG antibody concentration in serum and synovial fluid will be equivalent.

In some cases, it can be useful to perform a PCR for Borrelia using DNA from synovial fluid or from a biopsy fragment of the synovium (56).

If the clinical picture is suggestive of LB, but the serology is negative, the clinical symptoms should over-rule a negative test, as pointed out by Burgdorfer. Commercial test kits are often inaccurate and can give negative results even in advanced LB. A negative test does not demonstrate the absence of LB and further investigations are needed to rule out differential diagnoses, such as that for an autoimmune disease (57).

Neurological Symptoms

Involvement of the nervous system occurs in up to 15% of patients with untreated LB (58). A summary of the possible neurological manifestations in LB is reported in **Table 2**.

Headache is the most frequent symptom. Cranial nerve involvement may occur, particularly that of the facial nerve (80%). Facial paralysis is bilateral in 25% (59, 60). Paralysis of the III, IV, VI cranial nerve, and optic neuritis can be observed.

Among children in Europe, the most common manifestations are facial nerve palsy (about 55%) and lymphocytic meningitis (about 30%) (61).

Meningopolyneuritis (Garin-Bujadoux-Bannwarth) with radicular pain and sometimes paresis of extremities or the abdominal wall (62, 63), neurologic bladder (64), and paresthesia can be observed. Myelitis is a rare manifestation of LB; although monofocal or multifocal lesions of the cervical spinal cord (65) have been described, as well as lombosacral myelitis (66) and acute transverse myelitis.

Pseudo tumor cerebri associated with LB was first described in 1985 (67). Subsequently, other cases have been described mainly in children (68) and rarely in adults (69).

TABLE 2 | Neurological Involvement in LB.

Lymphocytic Meningitis	
Cranial neuritis	Facial palsy
	Cranial nerves palsies of III, IV, VI
	Optic neuritis and optic atrophy
Meningoradiculitis	Garin-Bujadoux-Bannwarth syndrome
Myelitis	Monofocal lesion
	Multifocal lesions
	Acute transverse Myelitis
Encephalitis	Loss of consciousness
	Speech disorders
	Recent cognitive disorders
	Affective disorders
Cerebral vasculitis	
Pseudo tumor cerebri	
Peripheral neuropathy	Chronic asymmetric neuropathy
	Small fiber neuropathy
Psychiatric disorders	States of anxiety
	Depression
	States of panic

Infection of the central nervous system is observed in 2–4% of Lyme neuroborreliosis, typically in the late or chronic stage of the disease (70). Encephalitis presents non-specific MRI findings of diffuse involvement of the brain parenchyma. Cerebral, cerebellar parenchyma, and thalami can be involved (71).

Neuroborreliosis can be associated with speech disorders, recent cognitive, and affective disorders (72), psychiatric disorders, states of anxiety, depression (73), and states of panic, and restless syndrome can be related to LB (74).

Cerebral vasculitis in patients with LB is observed in about 0.3% of cases (75). In some cases, the possibility of infection or co-infection (76) with *Borrelia miyamotoi*, which can be transmitted by the same tick as LB, should be considered (77, 78).

Neurological examination is suggested in order to rule out a differential diagnosis. In addition to the serological tests for anti-Borrelia antibodies by ELISA and Western Blot, it is also possible to perform a PCR for the detection of Borrelia DNA in cerebrospinal fluid (79) as well as an ELISA for Chemokine 13 (80).

Peripheral neuropathy can be detected in about 5–10% of Lyme neuroborreliosis cases. It can present as a chronic asymmetric neuropathy, usually without intrathecal antibodies (81).

For late neuroborreliosis, a careful examination is suggested for possible acrodermatitis chronica atrophicans (acral acrocyanotic appearance, and to verify any differences in limbs diameter) (82), and possibly a biopsy (for example on the ankle presenting neuropathic alterations) for histological examination of the small nervous fibers. Small fiber neuropathy (SFN) can be observed after antibiotic treatment (Post-treatment Lyme disease syndrome—PTLDS) and may be responsible for sensory symptoms (83).

In most patients, examination of the cerebrospinal fluid (CSF) reveals lymphocytic pleocytosis, damage to the blood-CSF-barrier, and an intrathecal synthesis of immunoglobulin IgM, IgG, and sometimes IgA (84); the protidorrachia is normal or slightly increased; the glycorrachia is normal or only slightly diminished.

During paralysis of the facial nerve, the CSF often presents lymphocytic pleocytosis even in the absence of signs and symptoms of meningitis (85).

After the onset of neurological symptoms, for a short time, intrathecal synthesis may not be detectable and CSF pleocytosis may be absent especially in children with isolated paralysis of the seventh cranial nerve (86). The production of intrathecal antibodies can continue even after recovery. On the other hand, intrathecal synthesis of specific antibodies is lacking in many patients with neuroborreliosis.

The use of chemokine (C–X–C motif) ligand 13 (CXCL13), a B-cell attracting chemokine, was debated for the laboratory diagnosis of acute Lyme neuroborreliosis in CSF (87). CXCL13 can be detected in CSF early in the disease and it has been reported to decrease with treatment (88). However, CXCL13 is not specific for Lyme neuroborreliosis and can also be found in some other inflammatory diseases of the CNS (88).

The different genospecies are often related to different clinical manifestations. *Borrelia garinii* is mainly related to typical early Lyme Neuroborreliosis (i.e., pain, meningoradiculoneuritis, or Bannwarth syndrome) while *Borrelia valaisiana* causes neurologic Lyme manifestations less frequently (89); *Borrelia afzelii* is less specific for neurologic manifestations as radicular pain and meningeal symptoms are rarely present (79). It is observed more often in late Neuroborreliosis by diffusion from the skin to small nerve fibers, often deriving from Acrodermatitis chronica atrophicans (82). It is able to cross the blood-brain barrier, but has a limited ability to produce inflammation in the CSF. The role of this genospecies has yet to be fully clarified.

Heart Symptoms

The involvement of the heart is observed in 4–10% of patients with LB, of whom 90% have Lyme carditis (90, 91). The most frequent manifestations are:

- Atrioventricular Conduction disorder or other rhythm disorders,
- Myocarditis (92, 93),
- Pericarditis (94),
- Postural Orthostatic Tachycardia Syndrome (POTS) (95).

In addition to dyspnea, chest pain, or irregular heartbeat, typical symptoms include syncope episodes (93). On physical examination, 35% of patients had bradycardia and about 15% tachycardia.

If heart involvement in LB is suspected, a cardiological examination is suggested. The following investigations should be addressed: 12-channel ECG and 24-h ECG Holter (query: rhythm analysis, PQ interval, QRS width, ectopic beats), chest X-ray (question: heart size, congestion); echocardiography (diameter, ejection fraction, abnormal wall movement, pericardial effusion); cardiac MRI, and in selected cases myocardial biopsy for histological examination and cultural isolation of Borrelia (96). Electrophysiological examination can be done only in selected cases to confirm the diagnosis and establish a prognosis, as it is a highly invasive procedure and can cause arrhythmia. Patients should be clearly informed about the procedure and its associated risk.

Ocular Symptoms

Ocular manifestations can be linked to a direct involvement of the eye or can be secondary to Neuroborreliosis. Ocular involvement, is possible at every stage of LB and they can be summarized as follows:

- Follicular conjunctivitis often self-limited, and,
- Photophobia.

They can appear in the first stages of LB.

In the early disseminated phase, these manifestations are possible:

- Macular edema,
- Uveitis and Iridocyclitis,
- Optic Neuritis and Neuroretinitis,
- Retinal Vasculitis and Choroiditis,
- Branch Retinal Vein Occlusion (BRVO) (97),
- White Dot Syndrome (98),
- Stromal Keratitis and Episcleritis.

Intermediate uveitis is the most common uveitis in LB. Posterior uveitis is mostly associated with chorioretinal involvement (99).

Keratitis is characteristic of the second and third stages of LB and may either be interstitial or ulcerative. Episcleritis and scleritis are rare and can be observed mainly in the late phase of LB (100).

Regarding ocular manifestations due to Neuroborreliosis, they include:

- Myositis of Extraocular Muscles,
- Facial Palsy and other Cranial Nerve Palsies (101),
- Horner's syndrome (102).

WHEN TO SUSPECT COINFECTIONS

Coinfections should be suspected in the following cases (103, 104):

- ✓ in the presence of fever and headache,
- ✓ in patients diagnosed with LB, who do not clinically improve or,
- ✓ whose symptoms have changed (e.g., appearance of febrile episodes) after adequate antibiotic treatment,
- ✓ when patients have leukopenia and neutropenia, persistent after treatment, or high ESR,
- ✓ when patients present purple, persistent skin lesions, even the same purpuric Erythema migrans (in our experience).

In these cases tests for Rickettsia, Anaplasma (105), Bartonella, Babesia (106), and TBE (FSME Frühsommer-Meningoenzephalitis) (105, 107) and Powassan virus (108) are suggested.

OCCASIONAL POSITIVITY OF ANTI-BORRELIA ANTIBODIES

The spirochetes may persist in affected organs even months to years after the initial infection, causing a chronic form of illness. Therefore, antimicrobial agents have been found to have a role in all stages of the disease (109).

When patients come to the Lyme Disease Center, because they have been found to be positive for anti-Borrelia antibodies, it is necessary to request an accurate medical history including the geographical area where the patient lives, recollection of a tick bite, and if applicable, the recollection of a circular rash, its possible location, and its duration. This collection of information should be followed by an accurate examination for the presence of LB related symptoms. Medical history should also include any previous antibiotic treatment.

In the absence of any reported tick bite or EM and related clinical manifestations, if the serological test results are positive in IgG antibodies it is recommended to perform a WB, whereas positive IgM may not be specific, and serology should be repeated after 6 months.

When the skin, the myo-articular system, and/or the nervous, cardiac or ocular systems are involved, specific investigations must be carried out, as indicated in the two previous paragraphs.

These patients should also be subjected to immunological testing, as Borrelia antigens can induce autoimmune diseases in predisposed subjects (Trigger Factor).

In some cases, Borrelia induces the production of antibodies against certain surface antigens, which cross-react with specific sequences of organism structures (antigenic camouflage). It is known, in fact, that there can be cross-reactivity between OspA and the human leukocyte function antigen (LFA) (110, 111), as well as between Osp and acetylcholine receptors, enolase gamma, and Borrelia Enolase (112).

A thorough diagnostic examination should be based on the clinical picture, the organs involved, the serological pattern, and the tests that have been already performed.

PERSISTENCE OF THE CLINICAL MANIFESTATIONS AFTER TREATMENT

The persistence of symptoms related to LB can be observed in untreated patients as well as in patients who have undergone treatment but continue to present symptoms. Untreated patients can develop persistent signs and symptoms, which usually involve the joints and less commonly the nervous system (113). Patients who instead have been treated mainly report a worsening of subjective symptoms. After 6 months, 36% of patients experienced an increase in fatigue, 20% complained of widespread pain, and 45% of neurocognitive impairment (114). Long-term persistent illness following antibiotic treatment is not uncommon, especially when treatment is delayed. About 10–20% of patients treated for early or late LB experience persistent symptoms, which may last for months or years (115). Symptoms consist of fatigue, joint and muscle pains, recent cognitive disorders, root pain, paresthesia, or dysesthesia. If we analyze the group of patients treated for Neuroborreliosis, this percentage increases significantly. Eikeland found that in Europe only 56% of patients treated with antibiotics for neuroborreliosis were symptom-free 30 months after treatment (116, 117).

Some published authors of medical research recognize mainly two clinical scenarios: the first characterized by typical symptoms of post-Lyme disease when symptoms persist for <6 months, and post-treatment Lyme disease syndrome or chronic Lyme disease if symptoms are debilitating and persist after treatment (118).

In the International Lyme and associated diseases society (ILADS) guidelines, "chronic Lyme disease" is described as a multisystem illness with persistent symptoms (119, 120), including fatigue, cognitive dysfunction, headaches, sleep disturbances, and other neurologic features, such as demyelinating disease, peripheral neuropathy, and sometimes motor neuron disease, neuropsychiatric presentations, cardiac presentations (including electrical conduction delays and dilated cardiomyopathy), and musculoskeletal problems (121–123). The cause may consist in residual damage to tissues and the immune system and cytokine production (122, 123), which occurs as a consequence of the infection causing possible modification of protein antigens located on the cell membrane. According to certain controlled studies, post-treatment Lyme disease syndrome (PTLDS) has often been shown to be non-responsive to antibiotic therapy. Several hypotheses have been suggested in order to explain PTLDS, among them, the presence of bacterial debris, autoimmunity, and co-infections, (120, 124, 125). In several studies, persistent Borrelia was isolated by culture or PCR (126–139).

The effectiveness of Ceftriaxone in several cases supports the hypothesis of bacterial persisters which survive in spite of previous antibiotic treatment (140). Delong et al. (140) have reported that retreatment can be effective, but further studies are needed to assess the role of antibiotics for persistent infection. It has been demonstrated that the persistence of *Borrelia burgdorferi* is likely due to the development of biologically less active permanent forms (Spheroblasts and round shapes) and of biofilm (141, 142). Biofilm analysis (Clinical Biofilm Ring Test—cBRT) (143) and treatment can produce an improvement in test results (144). In some cases, Borrelia can induce the production of antibodies against certain surface antigens, which cross-react with specific sequences of organism structures (antigenic camouflage). OspA is known, in fact, to cross-react with LFA, as well as Osp with Acetylcholine receptors. Treatment of *B. burgdorferi* in the stationary phase can result in a higher probability of regrowth once antibiotic treatment is interrupted (119).

Post-Treatment Lyme Disease Symptoms (PTLDS) and Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME) have several clinical features in common, including fatigue, musculoskeletal pain, and cognitive difficulties. The Canadian Clinical Criteria for CFS/ME diagnosis include the following symptoms: Fatigue > 6 months, limited physical activity, unrefreshing sleep, impaired thinking and speech, vertigo, post-exertional fatigue, stress induced by exertion, reduced concentration, orthostatic intolerance, food intolerance (145).

Immunologic mechanisms have been suspected to play a role in both PTLDS and CFS/ME.

In CFS/ME patients, serum Activin B levels were significantly elevated compared with control subjects. Elevated Activin B levels together with normal Activin A levels identified patients with the diagnostic symptoms of CFS/ME (146, 147).

It has also been hypothesized that there is an immunosignature specific to CFS/ME and that this could aid the diagnosis. Scientists were in fact able to identify a 256-peptide signature that separates CFS/ME samples from healthy controls (148).

An increase in levels and frequency of IgG anti-neural antibody reactivity has been found in PTLDS. The anti-neural antibody response was independent from serologic positivity for antibodies to *Borrelia burgdorferi*; however there was no significant difference in the prevalence of anti-neural antibody reactivity between CFS/ME patients and healthy controls (149).

PREGNANCY AND PEDIATRIC CASE ASSESSMENT

It is documented that trans-placental transmission of the spirochetes from the mother to the fetus is possible, and Borrelia starts crossing the placenta (150, 151) during the first month, unlike Treponema, which passes through the placenta barrier starting from the 5th month. A case of congenital Lyme with multiple annular erythema at birth has been reported in a child whose mother reported having an erythema migrans during pregnancy. Culture of skin biopsy from the child's skin lesion was positive for *Borrelia garinii* and rapid recovery was achieved after antibiotic therapy (152). A study on seven pregnant European women with EM and Borrelia isolated from blood indicated that the course and outcome of early LB was uneventful when pregnant women were treated with intravenous ceftriaxone, and that the outcome of their pregnancies was good (153). Therefore, in case of pregnancy, antibiotic prophylaxis treatment may be appropriate in the case of tick bites in endemic areas.

Below is a description of the symptoms of LB in children with potential exposure to tick bites, who have been diagnosed with EM or positive serological results or clinical manifestations compatible with LB.

Clinical suspicion of Lyme disease is based on the following clinical manifestations: for early localized LB, the presence of erythema migrans, often on the face, possibly associated with conjunctivitis and/or photophobia; for early disseminated LB the presence of multiple annular erythemas, Borrelial lymphocytoma, cranial neuritis, headache and/or pain and stiffness in the neck, migrant myo-arthralgia with possible involvement of the temporomandibular joint, alterations of electrocardiogram suggestive of carditis; for late BL the presence of arthritis. Acrodermatitis chronica atrophicans can also occur in children, but it is rare (154).

Patients with non-specific symptoms (e.g., fever or fatigue without specific manifestations of early, disseminated or late Lyme disease) are classified as probably not affected by Lyme disease. These patients should be considered positive only if, after 1 month, serology tests demonstrate serum conversion.

In some cases a rapid test response is required, ELISA or CLIA (155). Clinical evaluation plays a fundamental role when having to make initial decisions regarding children who visit the pediatric emergency room.

DETECTION OF BORRELIA IN CLINICAL SAMPLES

Indirect Methods of Borrelia Detection

Detection of Antibodies Against *Borrelia burgdorferi sensu lato* Complex

Several commercial products are available for detecting IgG and/or IgM antibodies against *Borrelia burgdorferi s.l.* complex. Test systems comprise different techniques including the Enzyme-linked immunosorbent assay (ELISA), the Enzyme-Immunoassay (EIA), the Enzyme-Linked Fluorescence Assay (ELFA), the Chemoluminescence Immunoassay (CLIA), Luminex, Fluoro-Immunoassay (FIA), and Western Blots/Immunoblots. Some tests use antigens obtained from native Borrelia bacteria, whilst others use manufacturing methods to prepare recombinant antigens. In some assays a mixture of both are used.

The European and North American guidelines indicate that the diagnosis of LB is currently based on a two-tier serology at all stages of the infection, except when erythema migrans is present (156). The two-tier testing procedure includes ELISA or EIA or VlsE/C6 as the first test and a Western Blot/Immunoblot assay as a confirmatory test. The VlsE Complex (variable major protein-like sequence Expressed—Vmp 35 kDa) is a surface protein formed by three defined domains: two invariable constant regions at the COOH and NH_2 terminals, and one internal variable region. The invariable, internal areas are masked and protected by the "*in vivo*" external variable regions. Due to the continuous modifications of its external antigenically variable component, Borrelia is able to escape the immune system.

After the death of the spirochetes, the VlsE protein is presented in its entirety to the immune system, which can thus induce the production of antibodies against the preserved and invariable regions of VlsE. The dosage of the VlsE protein and its sixth invariant region (IR6) peptide of *Borrelia burgdorferi* has been reported to quantitatively vary after antibiotic treatment (157–159), although VlsE and C6 are detected both in convalescent and healthy people, and thus they do not differentiate between active and past infection. OspC is used for detection of specific IgM antibodies in the first stage of the serologic test, either as a single antigen or as a mixture with other antigens.

Immunoblot (western blot) is generally used to confirm positivity and can characterize the immune responses to specific proteins *of Borrelia burgdorferi s.l.* complex. The test kit manufacturers clearly define the interpretation for positive, negative, and equivocal samples.

The European Union Concerted Action on Lyme Borreliosis/EUCALB has conducted a multicenter study for

the standardization of the interpretative criteria of immunoblot results in Europe. Although a set of eight bands were identified as significant in each participant laboratory, no single rule was formulated for use across Europe (160). The sensitivity of serological tests for diagnosis of LB is highly heterogeneous, varying with clinical manifestations (161). Average sensitivity estimates of 50% for erythema migrans, 77% for neuroborreliosis, 97% for acrodermatitis chronica atrophicans, and 73% for unspecified LB have been reported (162). Overall, the mean sensitivity of the serologic test was reported in a meta-analysis to be 59.5% (range: 30.6–86.2%) (163). Most European and North American guidelines recommend searching for intrathecal antibody production for the diagnosis of early Lyme neuroborreliosis (156).

In recent years, other commercially available serological tests have been developed for Borrelia detection. Among them, the TickPlex assay is an ELISA-based test, which also contains a new antigen for round bodies/persister forms of Borrelia. This assay has been reported to be useful in different stages of LB and the upgraded test also allows to simultaneously determine IgM and IgG antibodies of several tick-transmitted bacterial and viral pathogens (https://www.arminlabs.com/en/tests/tickplex).

Direct Detection of Borrelia

Direct detection of *B. burgdorferi sensu lato* can be achieved by culture of the infectious agent, by microscopy, and by the use of molecular methods for the detection of Borrelia nucleic acids. These methods vary in sensitivity and procedure complexity. They can provide evidence for the presence of intact spirochetes or spirochete components, such as DNA or protein, in tick vectors, reservoir hosts, or patients.

Culture

Although *in vitro* cultivation of *Borrelia* from clinical samples represents the golden standard for proving an active infection, this method cannot be routinely used for diagnosis as it is time consuming and has low clinical sensitivity (54, 164). *Borrelia burgdorferi sensu lato* culture can be obtained from various tissues and body fluids with variable yield using dedicated media, such as the modified Kelly-Pettenkofer medium (MKP), the Barbour-Stoenner-Kelly II (BSK-II) medium, and the commercially available BSK-H medium (165, 166). *Borrelia* cultivation from clinical samples is mostly successful from skin biopsy when compared to blood and CSF cultures (165, 167).

Microscopy

Borrelia burgdorferi sensu lato detection by light microscopy is not feasible in clinical practice. The low *Borrelia* load does not allow a direct recognition of the spirochetes in tissue slides for routine diagnostic procedures. However, for specific purposes, the Warthin-Starry's silver stain (168, 169) and more recently the focus floating microscopy (FFM) (170–173), which are light microscopy-based techniques, can be used to detect *Borrelia* in clinical tissues. In addition, Borrelia species were also detected by electron microscopy in human samples from myocardial tissues (174) and crystalline keratopathy (175).

PCR

Among molecular methods of detecting *Borrelia*'s nucleic acids, PCR-based methods are the most widely used for confirmation of *Borrelia* infection (167). However, *Borrelia* diagnosis continues to be very difficult, even by PCR (176). PCR sensitivity for *Borrelia* diagnosis is, indeed, highly variable, because of the multiple factors involved in its detectability by PCR. The type of starting material (blood, skin biopsies, cerebrospinal fluid, synovial fluid), the DNA extraction protocols, the possible use of systems for enrichment of microbial DNA, the PCR targets and PCR approach (nested PCR, real time PCR, digital PCR, PCR followed by hybridization, etc.) influence PCR sensitivity (167, 177, 178). The variability in specimens mentioned above and target amplification have also been found in the CE-IVD PCR assays developed for Borrelia detection (177). Low bacterial concentration is the main concern, and a further hypothesis regarding the possibility that during infection *Borrelia* invades the intracellular niche has been suggested (176). Moreover, different non-motile atypical morphologies of *B. burgdorferi* (s.l.) spirochetes have been reported. These include looped or ring-shaped forms, blebs, round bodies, and cell wall deficient forms; spirochete colonies or biofilm aggregates have also been described. The above-mentioned morphologies can impact Borrelia detectability by PCR. Biofilm busters to increase Borrelia load have been suggested for more accurate PCR tests (144). Borrelia PCR from skin biopsy from patients with ECM and ACA usually has a higher rate of positivity, but with large variation among studies (167). However, as the lesions are *per se* pathognomonic of LB, PCR is now only used for research purposes for those lesions. The diagnostic sensitivity of PCR in body fluids is highly variable, depending on the sample type, on the volume of the sample and on the contamination from PCR inhibitors (179). In synovial fluid, PCR for Borrelia detection is more sensitive than in blood and CSF (167). Borrelia targets for PCR must be genetically stable and should enable the detection of all pathogen of Borrelia species. They can be located on the chromosome or on plasmid DNA. The most frequent chromosomal targets that have been reported in clinical studies are flagellin (26, 164, 180–182), 16S rRNA gene (180, 183–185), the gene codifying for the 66 kDa protein (26, 56, 184, 185), while the most used plasmid target is OspA (56, 180, 183, 186–188), which has been also reported to be more stable after degradation of spirochetes (178). At present the major concern in Borrelia diagnosis by PCR is the lack of standardization of the protocols and analyzed targets (167, 177, 178). This heterogeneity in terms of PCR protocols and samples makes it difficult to diagnose LB unequivocally by PCR in settings in which the pre-test probability of LB is very low, including for instance patients suspected of late LB, with negative serology (178).

Novel Approaches in Borrelia Detection

Because of the limits of serology in detecting the *Borrelia sensu lato* complex in clinical samples, other commercially available tests have been developed. Among them, the T cell response tests, including the lymphocyte transformation test (LTT and MELISA) and the enzyme linked immuno-spot (EliSpot) test

have been commercialized. They are based on the detection in patients' blood of Borrelia-specific T-lymphocyte, notably the T helper lymphocytes, which are reported to circulate in the blood in detectable numbers only during an active immune response against Borrelia and to persist in a non-florid infection in lymphoid organs (189).

Alternative tests to the traditional serology and PCR for Borrelia detection have also been proposed. Among them, Luminex-based approaches for Borrelia detection have been reported. This multiplex- high-throughput technique was used for the simultaneous detection of the plasmid contents of different B. burgdorferi strains (10 Ag-Luminex technology) (190), but also to diagnose *Borrelia miyamotoi* in the serum of European patients (191) as well as for the simultaneous detection of 10 insect-borne pathogens, including Borrelia (192). An immuno-PCR (iPCR) assay, which takes advantage of the PCR properties to increase the sensitivity of standard ELISA (193), was also developed and evaluated for the detection of antibodies to the *B. burgdorferi* C6 peptide (194). Other approaches refer to the metabolic profiling for early Lyme disease (195) and the measurement of IFN-γ after incubating blood with *Borrelia* antigens. The latter method was reported to be potentially useful in the laboratory diagnosis of early Lyme disease, even after antibiotic treatment (196).

ETHICS STATEMENT

Written informed consent was obtained as part of the hospital procedures from the individuals and/or minor' legal guardian/next of kin for the publication of any potentially identifiable images included in this article.

AUTHOR CONTRIBUTIONS

GT managed the clinical aspect of the review, MR the section dedicated to serology, SB the section related to direct diagnosis go LB. All authors drafted and revised the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Symptom Clusters and Functional Impairment in Individuals Treated for Lyme Borreliosis

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Context: Persistent fatigue, pain, and neurocognitive impairment are common in individuals following treatment for Lyme borreliosis (LB). Poor sleep, depression, visual disturbance, and sensory neuropathies have also been reported. The cause of these symptoms is unclear, and widely accepted effective treatment strategies are lacking.

Objectives: To identify symptom clusters in people with persistent symptoms previously treated for LB and to examine the relationship between symptom severity and perceived disability.

Methods: This was a retrospective chart review of individuals with a history of treatment of LB referred to The Dean Center for Tick-Borne Illness at Spaulding Rehabilitation Hospital between 2015 and 2018 ($n = 270$) because of persistent symptoms. Symptoms and functional impairment were collected using the General Symptom Questionnaire-30 (GSQ-30), and the Sheehan Disability Scale. Clinical tests were conducted to evaluate for tick-borne co-infections and to rule out medical disorders that could mimic LB symptomatology. Exploratory factor analysis was performed to identify symptom clusters.

Results: Five symptom clusters were identified. Each cluster was assigned a name to reflect the possible underlying etiology and was based on the majority of the symptoms in the cluster: the neuropathy symptom cluster, sleep-fatigue symptom cluster, migraine symptom cluster, cognitive symptom cluster, and mood symptom cluster. Symptom severity for each symptom cluster was positively associated with global functional impairment ($p < 0.001$).

Conclusion: Identifying the interrelationship between symptoms in post-treatment LB in a cluster can aid in the identification of the etiological basis of these symptoms and could lead to more effective symptom management strategies.

Key Message: This article describes symptom clusters in individuals with a history of Lyme borreliosis. Five clusters were identified: sleep-fatigue, neuropathy, migraine-like, cognition, and mood clusters. Identifying the interrelationship between symptoms in each of the identified clusters could aid in more effective symptom management through identifying triggering symptoms or an underlying etiology.

Keywords: lyme borreliosis, symptom cluster, disability, fatigue, neurocognitive impairment

INTRODUCTION

Lyme borreliosis (LB) is caused by various tick-borne genospecies of the spirochete bacteria *Borrelia burgdorferi sensu lato* (1, 2) and is a multisystem, multi-stage disease. LB is the most common vector-borne illness in the US, and the number of cases has increased steadily over the last 25 years (3). Transmission of the *B. burgdorferi* spirochete from infected ticks to its human host begins with the translocation of *B. burgdorferi* from the gut to the salivary glands of infected ticks while feeding on its human host (4). At the time of initial presentation of LB, *erythema migrans* (commonly described as a "bullseye" rash or an expanding, homogeneously red rash), is observed in <60% of infected patients within 7–10 days at the site of the tick bite (5–8). The rash usually resolves within weeks, even in the absence of antibacterial therapy. Additional signs of acute disseminated LB include fever, fatigue, muscle and joint pain, headache, and lymphadenopathy (5). Notably, other tickborne infections such as Anaplasma and Ehrlichia can manifest with similar flu-like symptoms and when co-existing with *B.burgdorferi*, increase the severity of the presentation. If *erythema migrans* is absent at the onset of infection and the flu-like symptoms are presumed to be related to non-specific viral infection (9), LB can go undiagnosed and untreated for weeks, months, or even years. Importantly, the spirochete can enter the bloodstream and disseminate, often affecting the heart, joints, and nervous system (5). Lyme neuroborreliosis (LNB) is reported to occur in 10–15% of LB patients although this may be an underestimate, as *Borrelia burgdorferi* has been shown to disseminate to the central nervous system (CNS) very early in the course of acute disseminated infection with minimal if any clinical evidence of CNS involvement (10). Furthermore, a latent neuroborreliosis can exist for quite some time without significant symptoms, then present with late CNS involvement many months to years after initial infection and less characteristic symptoms (11). Symptoms may include facial paralysis and other cranial neuropathies, headache, neck stiffness, fatigue, paresthesias, meningeal signs, depression, anxiety disorders, peripheral nervous system problems, encephalitis or encephalomyelitis, chronic meningitis, and stroke secondary to cerebral vasculitis (11–18).

A subset of individuals with Lyme borreliosis go on to experience persistent or relapsing-remitting symptoms including fatigue, pain, and neurocognitive difficulties after treatment; an illness referred to as post-treatment Lyme disease syndrome (PTLDS). These chronic symptoms are of sufficient severity to impact quality of life and physical functioning (15, 19–23). The current definition of PTLDS, developed by the Infectious Diseases Society of America (IDSA), is clinician-documented Lyme borreliosis treated with standard antibiotic regimens, with onset of fatigue, widespread musculoskeletal pain, or cognitive difficulties within 6 months of Lyme disease diagnosis and with continuous or relapsing symptoms persisting for at least 6 months after treatment has ended (24). Risk factors for the development of persistent symptoms include a delay in diagnosis and treatment, the severity of the initial infection, incomplete recovery at 4-months post-treatment, and a history of relapse (15, 21, 22, 25, 26).

While mounting scientific evidence in the last decade points to potential persistence of the bacterium *Borrelia* after antibiotic treatment, *in vitro* and *in vivo* (27–32), there exists ongoing confusion and controversy in the literature around PTLDS symptoms, including their etiology and management. The benefits of additional antibiotic therapy for PTLDS have been debated. Significant gains in certain domains have been reported in open label prospective studies utilizing extended antibiotic courses (33, 34), as well as two of the four randomized controlled trials of regimens containing intravenous ceftriaxone (20, 35–37); however, the authors of both randomized controlled trials that found gains in select domains with intravenous ceftriaxone therapy ultimately concluded that their studies did not support general use of IV ceftriaxone for PTLDS (35, 36). Although statistically significant improvements were seen in certain domains, their conclusions were based on risks of treatment as well as—in one trial—the lack of sustained benefit in cognitive improvement after completion of therapy (36), or—in the other—the benefit being limited to sustained improvement in a single domain, fatigue, which despite being a primary outcome measure, was deemed a "nonspecific" symptom (35). Issues surrounding the design of the randomized controlled trials and interpretation of their results have been debated (38–40). Importantly, studies to date have not led to comprehensive consensus guidelines for diagnosis and management of PTLDS. This underscores the need to more fully characterize its varied symptoms with the aim of better understanding potential underlying mechanisms which, in turn, can help inform management decisions.

While continuous or remitting fatigue, musculoskeletal pain, and/or cognitive difficulties are predominant, patients with persistent symptoms following LB treatment frequently report a variety of other symptoms including poor sleep, depression, visual disturbance, and sensory neuropathies that can be similarly burdensome and may affect fitness and function (41, 42). The cause of these persistent symptoms is not known, although

several mechanisms have been proposed, including the direct neurotoxic effects of the spirochete, neuroinflammation, or autoimmunity (4, 43–48).

A symptom cluster is defined as a group of two or more symptoms that co-occur and are interrelated (49). The identification of symptom clusters has been used extensively in chronic conditions, including cancer (50, 51), inflammatory bowel disease (52), chronic obstructive pulmonary disease (53), and multiple sclerosis (54), in which symptoms seldom occur individually. Identifying the interrelationship between symptoms in a cluster can aid in more effective symptom management. For instance, symptoms may cluster together through a shared etiology such as neuroinflammation (55) or because they share the same triggering symptom (56). Identifying symptom clusters in individuals who report persistent symptoms following treatment for LB could aid in more effective symptom management through identifying triggering symptoms or an underlying etiology. The purpose of this study was to identify symptom clusters in individuals with persistent symptoms following treatment for LB and to examine the relationship between symptom severity and perceived disability in this population.

MATERIALS AND METHODS

Cases

A retrospective chart review was conducted to examine symptoms and disability in individuals with a history of treatment for LB who were referred to The Dean Center for Tick-Borne Illness at Spaulding Rehabilitation Hospital in Boston. At the Dean Center, all patients completed symptom and disability surveys, which were incorporated into their medical chart. In addition, all patients underwent a complete blood count (CBC) and chemistry, tests of kidney, liver, thyroid function, and HgBA1c to rule out disorders that could mimic post-treatment Lyme borreliosis symptomatology (i.e., hypothyroidism, anemia, diabetes, etc.). Patients had serological testing for co-infections that are known or postulated to be tick-borne (i.e., Babesia, Anaplsama, Ehrlichia, Rickettsia, and Bartonella), either through their referring physician or at our center, and those with evidence of infection were treated according to established clinical protocols. The analysis of co-infection data have been omitted from this report and will be the subject of a separate study. Between 2015 and 2018, two-hundred and seventy adults ($\geq$18 years) were identified by medical chart review. The Institutional Review Board approved this retrospective chart review, and data was de-identified prior to analysis. Responsible Conduct of Research (National Institutes of Health; Massachusetts General Hospital) and the Health Insurance Portability and Accountability Act of 1996 Privacy Rule were observed.

Self-Report Symptoms and Functional Impairment

At the time of the first clinic encounter, each patient completed the 30-item General Symptom Questionnaire-30 (GSQ-30) which assesses symptom burden over the past 2 weeks (57) on a 0 to 4 scale where 0 = not at all, 1 = a little bit, 2 = somewhat, 3 = quite a bit, and 4 = very much. Patients also indicated whether any of the symptoms impaired their work, social, or family functioning, and if yes, which symptom was the most impairing. The GSQ-30 has shown excellent validity and internal consistency (57). The Sheehan Disability Scale (SDS) was also administered at the same time. The SDS is a widely used assessment of function in three domains: work/school, social life/leisure activities, and family life/home responsibilities (58). Each domain is scored using a 0–10 scale where 0 = not at all and 10 = extremely. The three domains are summed into a single-dimensional measure of global functional impairment with a range from 0 (no impairment) to 30 (highly impaired).

Statistical Analyses

All statistical analyses were performed using IBM SPSS version 24.0 (IBM) and R version 3.6.1. Descriptive statistics and frequency distributions were calculated for demographic and clinical characteristics. We used the standard Cronbachs α coefficient to determine reliability. We used exploratory factor analysis (EFA) with principal axis factoring to identify factors or "symptom clusters." The key concept of EFA is that multiple items on the GSQ have similar patterns of responses across individuals because they are all associated with a latent (i.e., not directly measured) variable. Principal axis factoring with oblique rotation (Varimax) was used as the factor model with squared multiple correlations used to establish communalities. The Kaiser-Meyer-Olkin test, a measure of how suited our dataset was for EFA, verified the sampling adequacy for the analysis; KMO = 0.922. The number of factors was determined using a scree plot and the total percentage of variance explained by each factor with an eigenvalue greater than the average eigenvalue. A factor loading $\geq$0.4 was used to identify significant factors, with at least two items loaded in each cluster (59). As symptoms are complex and could be cross-loaded on more than one factor, the decision to retain the symptom on one factor was based on the significance of the loading and the conceptual and clinical relevance of the symptom. Each factor orsymptom cluster was assigned a name to reflect the possible underlying etiology. Three items were removed from the EFA due to insufficient variation in the occurrence of these symptoms: shortness of breath, feeling feverish, and sweats, and/or chills.

RESULTS

Two-hundred and seventy adult cases were identified, of which 67.8% were female, with a mean age of 49 $\pm$ 14.8 years (Range 18–88) and 16.1 $\pm$ 1.4 years of education. For employment status, 12.7% were on disability or unemployed, 8.5% were retired, 6.8% were students, 2.5% were homemakers, and 69.6% were employed. The mean time since LB diagnosis and treatment was 10 $\pm$ 8.2 years (Range 1–43 years, median = 8 years).

Table 1 shows the mean symptom severity scores and symptom rankings based on responses to each of the 30 questions on the GSQ-30. Potential scores ranged from 0 (Not at all) to 4 (Very much). The mean symptom severity score range for the total study population was 0.7 $\pm$ 1.1 for "*feeling feverish*" to

TABLE 1 | Symptom severity and impairment scores.

Symptom[a]	Rank	Severity[b]		Impaired[c]
		Mean	SD	%
Feeling fatigued or having low energy	1	2.9	1.3	20
Muscle aches and pains	2	2.5	1.3	7.9
Not feeling rested upon wakening	3	2.5	1.5	1.4
Trouble with memory	4	2.4	1.3	6.0
Feeling worse after normal physical exertion	5	2.4	1.5	2.3
Slower speed of thinking	6	2.4	1.4	10.2
Trouble finding words or retrieving names	7	2.3	1.4	4.7
Needing more sleep than usual	8	2.2	1.5	1.4
Trouble falling or staying asleep	9	2.2	1.5	2.3
Joint pain or swelling	10	2.2	1.5	6.5
Stiff or painful neck	11	2.1	1.5	1.9
Muscle weakness	12	2.1	1.4	2.8
Back pain	13	2.1	1.5	6.0
Numbness or tingling	14	1.9	1.5	0.9
Headaches	15	1.9	1.4	5.1
Feeling irritable, sad, or decreased pleasure	16	1.9	1.4	2.8
Feeling panicky, anxious, or worried	17	1.8	1.5	3.7
Shooting, stabbing or burning pains	18	1.8	1.5	0.9
Change in visual clarity or trouble focusing	19	1.8	1.5	0.9
Discomfort with normal light or sound	20	1.7	1.5	2.3
Balance problems or sense of room spinning	21	1.7	1.5	2.3
Skin or muscle twitching	22	1.6	1.5	0.9
Hot or cold sensations in extremities	23	1.6	1.5	0.5
Light headed or uncomfortable on standing	24	1.6	1.4	0.5
Sweats and/or chills	25	1.4	1.3	1.9
Bladder discomfort or change in urination	26	1.2	1.4	-
Irregular or rapid heart beats	27	1.1	1.3	0.9
Nausea and/or vomiting	28	1.1	1.3	1.4
Shortness of breath	29	1.0	1.1	1.4
Feeling feverish	30	0.7	1.1	-

SD is standard deviation.

[a]Patients were asked how much they had been bothered by each of the symptoms listed during the past 2 weeks.

[b]Symptom severity: 0 = not at all, 1 = a little bit, 2 = somewhat, 3 = quite a bit, 4 = very much.

[c]% of total respondents (N = 215) who identified symptom as most impairing on work, social, or family functioning.

2.9 ± 1.3 for "*feeling fatigued or having low energy*." Over 80% ($n = 220$) of patients reported "yes" when asked whether any of the symptoms impaired work, social or family function. The top five symptoms identified as the greatest cause of impaired work, social, or family function, making up over 50% of respondents, were feeling fatigued or having low energy, slower speed of thinking, muscle aches or pains, joint pain or swelling, and trouble with memory (**Table 1**).

Figure 1 shows results from the exploratory factor analysis for responses on the GSQ-30 symptom survey in all patients. The six items in factor 1 (balance problems, discomfort with normal light and sound, nausea and/or vomiting, etc.) were called the migraine-like symptom cluster. The six items in factor 2 (feeling fatigued or having low energy, needing more sleep than usual, etc.) were called the sleep-fatigue symptom cluster. The eight symptoms in Factor 3 (i.e., muscle aches and pain, numbness and tingling, shooting, stabbing and burning pains, etc.) were called the neuropathy symptom cluster. The three symptoms in factor 4- trouble with memory, slower speed of thinking, and trouble finding words or retrieving names- were called the cognitive symptom cluster. Finally, the two items in factor 5, feeling panicky, anxious, or worried and feeling irritable, sad, or decreased pleasure, were called the mood symptom cluster. **Figure 2** shows the percentage of patients who reported being bothered by symptoms in each of the five symptom clusters ranging from "not at all" to "very much." Approximately 45% of patients reported that they were troubled quite a bit or very much by fatigue or cognitive difficulties. Mood symptoms were the next most troubling, with approximately 30% of patients reporting that they were bothered quite a bit of very much by these symptoms. Although migraine-like and neuropathic symptoms were the least troublesome, they were still troubling for approximately 20% of patients.

TABLE 2 | Associations between symptom severity and global disability score.

Symptom cluster	β Coefficient (95% CI)
Neuropathy	0.46 (2.59–4.38)*
Fatigue-Sleep	0.57 (3.43–5.05)*
Migraine-like	0.54 (3.43–5.22)*
Cognition	0.44 (2.10–3.68)*
Mood	0.44 (2.28–3.73)*

*$p < 0.001$.

SDS data was available for 220 patients. Mean scores on the SDS work/school, social life/ leisure activities, and family life/home responsibilities domains were 5.7 ± 3.5, 6.5 ± 3.0, and 6.2 ± 3.1, respectively. The mean Global Functional Impairment score was 18.2 ± 8.9. Increasing symptom severity for each symptom cluster was linearly associated with greater global disability ($p < 0.001$, **Table 2**). Functional impairment increased when the severity of fatigue, cognitive, mood, and migraine-like symptoms increased from moderate to severe, and when neuropathy symptoms increased from mild to moderate (**Figure 3**).

DISCUSSION

This is the first study to examine symptom clusters in a large cohort of individuals with persistent symptoms following treatment for Lyme borreliosis. The identification of symptom clusters may help us to identify mechanisms, and allow us to correlate clusters to specific infectious agents. We identified five symptom clusters, which we named the neuropathy, sleep-fatigue, migraine-like, cognitive, and mood symptom clusters. The sleep-fatigue symptom cluster included three items related to sleep quality: needing more sleep than usual, not feeling rested upon awakening, and trouble falling or staying asleep. Prior studies have reported poorer sleep in individuals with

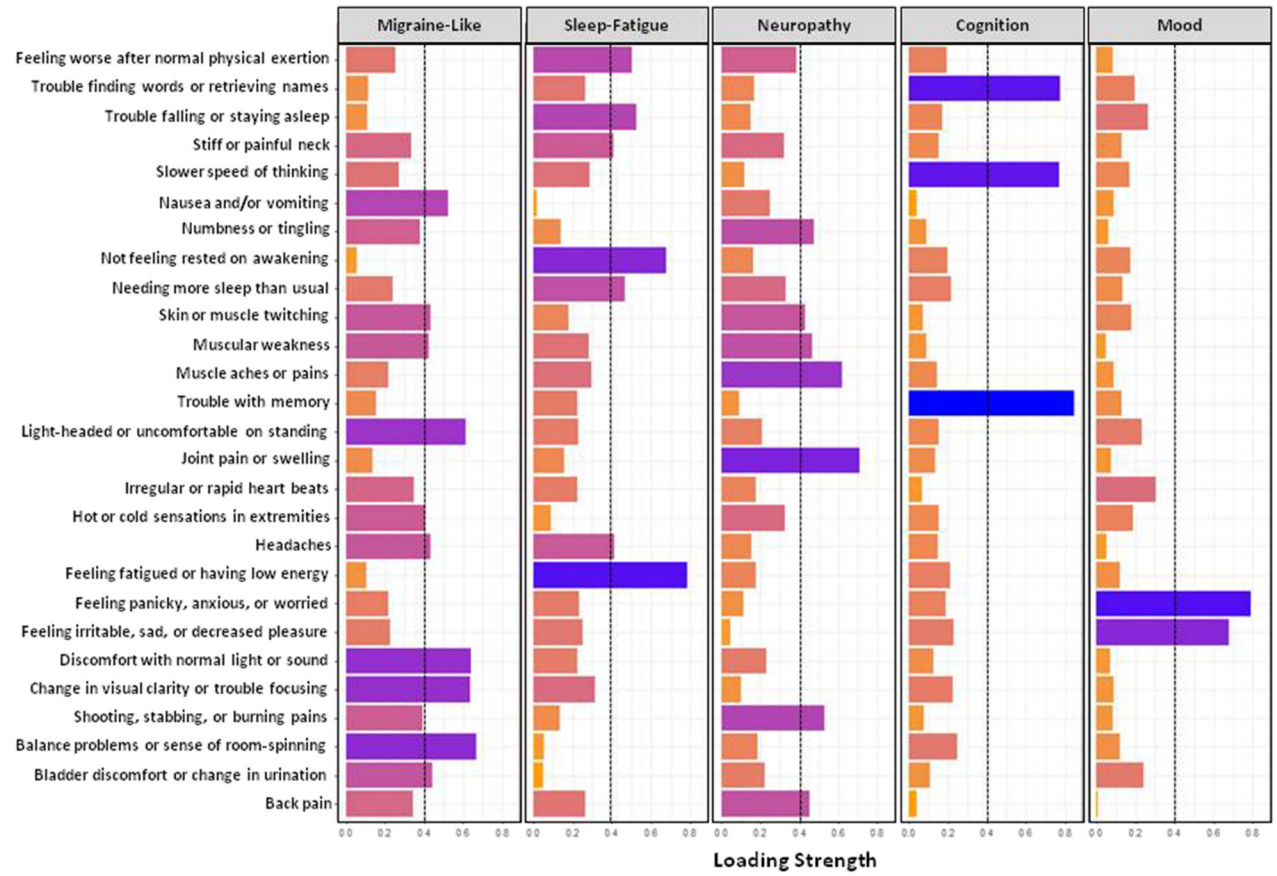

FIGURE 1 | Exploratory factor analysis using scores on the GSQ-30.

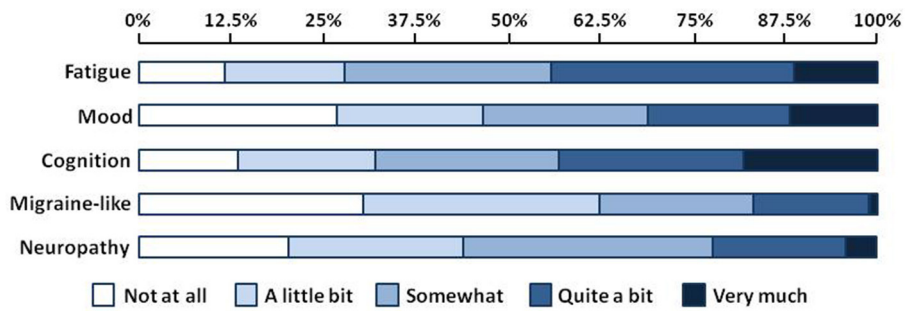

FIGURE 2 | % of patients reporting being bothered by symptoms in each symptoms clusters.

PTLDS compared to healthy controls (41, 60, 61), and self-reported fatigue and perceived poor sleep quality frequently co-occur in other chronic conditions, including cancer (62), diabetes (60, 63), and chronic fatigue syndrome (64). While there are frequent reports of sleep disruption in LB, only one study has examined both patient-reported and objectively measured sleep outcomes in this population. Greenberg et al. compared self-reported and polysomnographic assessment of sleep quality in LB patients and in matched healthy controls (65). Compared to healthy controls, LB patients reported greater difficulty falling asleep, more restless sleep, and increased daytime sleepiness (65). Objective sleep assessment revealed an increase in onset latency (time to fall asleep), decreased sleep efficiency (time in actual sleep divided by time attempting to sleep), and higher frequency of awakenings (65). Poor sleep quality could be the triggering symptom in this cluster since

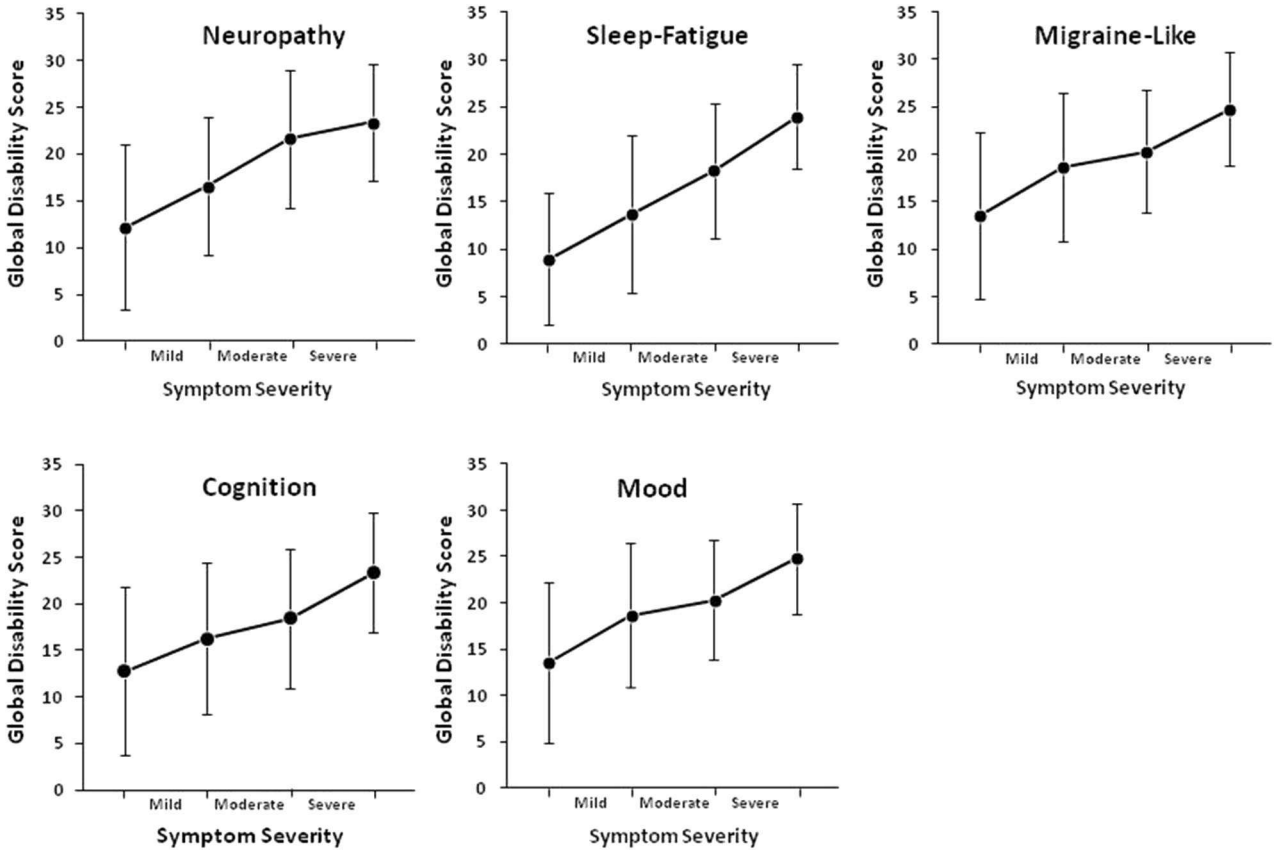

FIGURE 3 | Effect of increasing symptoms severity of functional impairment.

inadequate sleep would likely increase fatigue. Alternatively, individuals with high levels of fatigue, in particular, daytime sleepiness, could resort to daytime napping, which, in turn, could lead to fragmented and non-refreshing sleep at night. Further characterization of sleep deficits in people with persistent symptoms post LB are needed to develop strategies aimed at improving sleep and possibly fatigue in LB patients with documented sleep deficits.

The majority of the symptoms in the neuropathy symptom cluster were related to musculoskeletal pain and weakness, paresthesia, and hot and cold sensation in the extremities, symptoms common in small fiber neuropathy (SFN). In a recent study, Novak et al. examined SFN in individuals with persistent LB symptoms and found abnormal epidermal nerve fiber density (ENFD) in 90%, abnormal sweat gland nerve fiber density (SGNFD) in 50%, and both ENFD and SGNFD in 40% (42). Consistent with these sensorimotor deficits, many LB patients have been shown to have a reduced vibration threshold in their extremities. In the study by Rebman et al., approximately 30% of LB patients had scores below the age-adjusted cutoffs for vibration threshold in upper and lower extremities (41). Despite evidence of somatosensory deficits in the lower extremities, there have been no studies to date that have performed a detailed examination of gait and balance deficits in LB. Although the mechanism whereby LB causes SFN is not known, several mechanisms of neuronal injury have been proposed, including the direct neurotoxic effects of the spirochete, neuroinflammation, or autoimmunity (4, 43, 46, 48).

Neuroinflammatory processes may also underlie the symptoms found in the migraine symptom cluster, which included items related to visual disturbances, sensitivity to light or sound, balance problems, or being lightheaded or uncomfortable while standing. Indeed, we labeled this symptom cluster the migraine-like cluster because these symptoms are common in migraine. The overlap between migraine symptoms and those of post-treatment LB suggests that they may share a common mechanism. Migraine is a chronic neurological disorder that affects the central and peripheral nervous systems (66). Altered activity of the thalamic and thalamo-cortical areas contribute to aberrant sensory processing inherent in migraine, while MRI studies have demonstrated altered connectivity in a number of brain regions, including the cerebellum, hypothalamus, and brain stem (66). Recently the neurogenic inflammatory mediator calcitonin gene-related peptide (CGRP) has been implicated in the etiology of migraine

(67). CGRP is produced by neurons in the CNS and the peripheral nervous system, where it acts as a vasodilator and inflammatory mediator acting via NF-κB (68–70). CGRP is released from neurons in response to a variety of environmental stimuli, including infectious agents such as *B. burgdorferi* (71). Consistent with its role in the etiology of migraine, individuals with acute migraine have elevated circulating levels of CGRP (72), and newly developed therapeutic monoclonal antibodies that inactivate circulating CGRP have proven efficacy in the treatment and prevention of migraine (67). Whether the same tools used to understand the cause of migraine could be applied to identify the cause of migraine-like symptoms in LB remains to be seen but could be a fruitful avenue for future investigation.

Items in the cognition symptom cluster included trouble with memory, trouble finding words or retrieving names, and slower speed of thinking. Self-reported cognitive deficits are frequently reported in LB and in other neurological and inflammatory conditions, including cancer, rheumatoid arthritis, and multiple sclerosis. However, self-reported cognitive deficits do not always correlate with performance on neuropsychological tests. Berende et al. found no association between self-reported cognitive difficulties in over two hundred LB patients and performance on objective tests of episodic memory, working memory / attention, verbal fluency, information-processing speed, and executive function (73). Less than 3% of participants had cognitive deficits based on neuropsychological testing, a rate comparable to the general population. Their findings were similar to the study by Kaplan et al. who similarly found no association between subjective cognitive difficulties and performance on tests of memory, attention, and executive functioning in 129 individuals with physician documented LB (74). Touradji et al. found that while over 90% of 124 LB patients reported cognitive difficulties, only 26% showed evidence of mild cognitive deficits in memory and processing speed (75). In contrast, Tager et al. reported significantly more objective cognitive deficits and psychiatric disturbances in children who developed new-onset cognitive complaints after Lyme disease compared with matched healthy controls (76). Objective cognitive deficits on neuropsychological evaluation, which included disturbances in visual and auditory processing and attention as well as in working memory and mental tracking, were still found after controlling for anxiety, depression and fatigue (76). Similarly, Keilp et al. observed statistically significant differences in several cognitive tests including tests of verbal comprehension, attention, executive function, working memory, and processing speed between patients with a history of LB and healthy controls (77). Like the Touradji study, Keilp et al. noted that the observed cognitive deficits were mild. Discordance between self-report and objectively measured cognitive function is not unique to LB and may reflect limitations in current neuropsychological testing which do not incorporate "real-world" demands on cognitive function. In the real world setting, cognitive tasks are frequently performed concurrently with motor tasks (i.e., walking while talking). While dual-tasking (i.e., performing cognitive and motor tasks concurrently) poses little problem for people with intact cognitive and sensorimotor function, it can be problematic for individuals with cognitive or motor deficits such as older adults and those with neurological conditions such as diabetes, stroke, or multiple sclerosis in which balance and cognitive deficits frequently co-exist (78–82). Given the reported sensorimotor deficits in LB patients, testing performance on cognitive tasks with a concurrent motor task may be a better indicator of cognitive decline than performance on a cognitive task administered alone under laboratory conditions. Further work is needed to fully understand the scope of neurocognitive problems in LB patients and to identify neural pathways that contribute to these deficits.

Finally, the two items in the mood symptom cluster- feeling panicky, anxious, or worried and feeling irritable, sad, or having decreased pleasure- are common symptoms of anxiety and depression. Symptoms of irritability and depression have been documented in LB (83–89) although it is unclear whether these symptoms are of sufficient severity to meet criteria for clinical anxiety/depression (89). A prior population-based retrospective cohort study did not show increased rates of depression in individuals with a history of LB and persistent symptoms compared to those without symptoms (19).

There are several limitations to the current study, including its retrospective, cross-sectional design. Data used in the analyses were extracted from the medical record and therefore lacked consistently documented clinical and demographic information that would typically be collected in a prospective research study. Although all patients seen at the clinic had a history of treatment for LB and were referred because of lingering symptoms, their charts lacked several pieces of information needed to determine whether they met the criteria for a diagnosis of PTLDS. Future studies should include a detailed analysis of the infectious origins of symptoms in these patients (i.e., multiplex PCR analysis combined with serology), which would allow us to determine whether specific clusters correlate to a particular infectious organism, or allow differential diagnoses. Because only clinical data from the initial clinic visit was used to create the symptom clusters, the stability of these clusters over time is not known. Future studies are needed to identify shared or distinct mechanisms, including distinct infectious organisms, that underlie these symptom clusters, which will aid in the development of new treatment strategies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Spaulding Rehabilitation Hospital Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

NZ, CM, EB, and LW had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. LW and EB conducted the statistical analyses. LW and NZ take responsibility for the study's concept and design. LW, NZ, CM, and EB participated in the drafting of the manuscript. NZ and DC obtained funding. QW and RO provided administrative, technical, or material support. All authors contributed to the acquisition, analysis or interpretation of the data and all contributed to critical revision of the manuscript for important intellectual content.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Borrelia burgdorferi-Induced Changes in the Class II Self-Immunopeptidome Displayed on HLA-DR Molecules Expressed by Dendritic Cells

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The MHC class II antigen processing and presentation pathway has evolved to derive short amino acid peptides from proteins that enter the endocytic pathway, load them onto MHC class II molecules and display them on the surface of antigen presenting cells for recognition by $CD4^+$ T cells. Under normal circumstances, peptides bound to MHC class II molecules are derived from host (self) proteins and not recognized by T cells due to tolerance mechanisms. Pathogens induce significant changes in the biology of antigen presenting cells, including upregulation of MHC processing and presentation. We therefore hypothesized that exposure to pathogens may alter the repertoire of self-peptides bound to MHC class II molecules. To test this hypothesis, we isolated monocyte-derived dendritic cells from healthy subjects, exposed them to the TLR-2 agonist lipoteichoic acid or live *Borrelia burgdorferi*, the causative agent of Lyme disease, and isolated and characterized HLA-DR associated peptides using mass spectrometry. Our results show that lipoteichoic acid-stimulated, *B. burgdorferi*-stimulated and unstimulated monocyte-derived dendritic cells largely derive their self-peptides from similar overlapping sets of host proteins. However, lipoteichoic acid and *B. burgdorferi* stimulation promote the processing and presentation of new sets of HLA-DR associated self-peptides derived from unique protein sources. Examination of processes and compartments these proteins reside in, indicate that activation of monocyte-derived dendritic cells changes the range of host self-proteins available for processing and presentation on MHC class II molecules. These findings reveal that the HLA-DR-bound self-immunopeptidome presented by mo-DCs is dynamic in nature and changes with activation state reflective of cellular function. In addition, among the repertoire of self-peptides bound to HLA-DR are several epitopes known to be recognized by autoreactive T cells. These studies are relevant to our basic understanding of pathogen-induced changes in monocyte-derived dendritic cell function, and the mechanisms involved in infection-induced autoimmune illnesses such as Lyme arthritis.

Keywords: Borrelia, immunopeptidome, HLA-DR, dendritic cells, MHC class II

INTRODUCTION

Lyme disease is an inflammatory illness initiated by infection with the *Borrelia burgdorferi* spirochete following a bite from an infected *Ixodes* tick (1). Over the last four decades, the number of Lyme disease cases has risen sharply, and it is now the most common vector-borne disease in the United States with over 300,000 cases each year (2). Symptoms of early Lyme disease can range from erythema migrans alone to systemic toxicity with signs of disseminated infection. A number of patients with undetected and untreated early Lyme disease will develop late-onset musculoskeletal (Lyme arthritis) or neurological symptoms (Neuroborreliosis) (3). While the acute infection and late-onset disease can be controlled by antibiotic therapy, in a subset of patients, arthritis with inflammation can be antibiotic-refractory (4). This outcome has been termed post-infectious Lyme arthritis, with autoimmune processes presumed to play a major role and although controversial, bacterial persistence cannot be excluded from contributing to the development of the illness (5). Further, 10–20% of patients treated for early Lyme disease develop Post-Treatment Lyme Disease Syndrome (PTLDS), a condition with unknown pathophysiology that may have an autoimmune component (6–8). Clearly, infection with *B. burgdorferi* triggers poorly understood immune processes, and considering the rising incidence of Lyme disease as well as the complexity of disease outcomes, a deeper understanding of the immune-mediated process triggered by Borrelia is needed.

Dendritic cells (DCs) are major drivers of the adaptive immune response against pathogens (9). These cells are present at strategic sites of pathogen entry such as the skin (10). At homeostasis, immature DCs are tissue resident, highly phagocytic, and have a constitutive antigen processing and presentation pathway expressing low levels of major histocompatibility (MHC) molecules, loaded with self-peptides. Upon encounter with a pathogen, a range of surface and intracellular pattern recognition receptors signal a complex maturation program, which leads in part, to the down regulation of phagocytic activity, the upregulation of relevant co-stimulatory molecules, as well as an increase in antigen processing, presentation, and MHC molecule expression (11). This results in a mature dendritic cell that expresses MHC molecules loaded with pathogen-derived peptides (11). These mature cells then relocate to the T cell rich areas of draining lymph nodes initiating a T cell dependent host response (12).

The infection route *via* tick bite introduces the Borrelia bacterium into the skin, a site where dendritic cells reside (10). Given the importance of DCs in the initiation of T cell-dependent responses and the key role for $CD4^+$effector T cells in human Lyme disease pathogenesis, we investigated the consequences of *B. burgdorferi* interaction with human monocyte-derived dendritic cells (mo-DCs) *in vitro*. The results show that *B. burgdorferi* uniquely alters the self-peptide repertoire. These peptides are derived from a new set of proteins which occupy novel compartments and/or pathways. These observations will be discussed in the context of human Lyme pathogenesis and infection-induced immunity.

MATERIALS AND METHODS

Borrelia burgdorferi Culture

Borrelia burgdorferi strains A3 (kindly provided by Dr. Utpal Pal, University of Maryland), B31 (ATCC 35210), and B31-5A19 (kindly provided by Dr. Monica Embers, Tulane University) were grown from frozen stocks in 5 mL tightly closed conical tubes of BSK-II incomplete culture medium made in house [9.82 g CMRL-1066 without L-glutamine, 5.0 g neopeptone, 2.0 g yeastolate, 6.0 g HEPES, 5.0 g glucose, 0.7 g sodium citrate dihydrate, 0.8 g sodium pyruvate, 0.4 g N-acetyl-D-glucosamine, 2.2 g sodium bicarbonate, 50.0 g bovine serum faction V (albumin, bovine serum), 1 L of deionized water], supplemented with 6% rabbit serum and 7% gelatin for 14 days. Bacterial cultures were grown at 34°C, 5% CO_2, and >95% humidity and used between 14 and 21 days old while in the logarithmic growth phase. For inoculum preparation, 1–5 mL of each *B. burgdorferi* strain was pelleted at 6000 RCF for 8 min at room temperature. Aliquots were resuspended in 1 mL of phosphate buffered saline (PBS), bacteria were counted using dark-field microscopy, pelleted and resuspended to the desired concentration in RPMI 1640 medium with 10% fetal bovine serum.

Monocyte-Derived Dendritic Cell Culture

Deidentified leukopacks classified as medical excess from consented, healthy, anonymous plasma/platelet donors were sourced from the Ann Arundel Medical Center in Annapolis, MD. Immunogenetic genotype information from the donor cohort was performed by the Johns Hopkins Immunogenetics Laboratory and is available in **Table 1**.

Peripheral blood mononuclear cells (PBMCs) were isolated from donor leukopacks by Ficoll-Paque density gradient centrifugation from ∼100 mL of leukocytes isolated by leukapheresis. CD14 MicroBeads (MACS Miltenyi Biotec) were used for $CD14^+$ selection according to manufacturer's instructions and isolated monocytes were differentiated into mo-DCs in Mo-DC Differentiation Medium (MACS Miltenyi Biotec) at 10^6 cells/ml for 7 days as described previously (13). Cells were incubated at 37°C, 5% CO_2, and >95% humidity. At the end of the culture period the resulting cell population was routinely >95% $CD14^-/CD11c^+$, as assessed by flow cytometry.

In vitro Activation of Monocyte-Derived Dendritic Cells for Flow Cytometry Analysis

We performed a natural antigen processing assay (NAPA) as described previously (13). Briefly, immature mo-DCs were seeded in 6-well plates at a density of 1×10^6 cells in 1 mL of RPMI 1640 and 10% fetal bovine serum, supplemented with 2 mM L-glutamine and 20 μM ß-mercaptoethanol in duplicate. Immature mo-DCs were left at rest or stimulated with 1 μg/mL purified lipoteichoic acid (LTA) from *Staphylococcus aureus* (InvivoGen, San Diego, CA) or live *B. burgdorferi* strains A3 or B31 at multiplicities of infection of 1 or 10 bacteria per cell for 8 and 24 h. Cells were incubated at 37°C, 5% CO_2, and >95% humidity. At the end of the incubation period, all samples were harvested into a microcentrifuge tube, pelleted at 1,200 rpm at room temperature for 3 min. Cells were washed with PBS/EDTA

TABLE 1 | Healthy donor identifiers, experimental details, and statistical results generated with PEAKS X from individual LC-MS/MS experiments.

Healthy Donor	Stimulus	Peptide-Spectrum Matches	Peptide Sequences	Proteins	Peptide −10lgP	False Discovery Rate (%)	HLA-DRB1 Locus	
							Allele 1	Allele 2
190125	None	755	464	451	≥20	2.60	N/A	N/A
	Lipoteichoic acid	N/A	N/A	N/A	N/A	N/A		
	Borrelia burgdorferi	2001	987	1081	≥20	0.90		
190506	None	572	411	442	≥20	0	DRB1*03:01/ 03:147	DRB1*03:02
	Lipoteichoic acid	601	440	546	≥20	0.2		
	Borrelia burgdorferi	1045	725	657	≥20	0.4		
190529	None	1439	731	671	≥20	0.3	DRB1*11:01	DRB1*04:01
	Lipoteichoic acid	1920	1205	1045	≥20	0.5		
	Borrelia burgdorferi	1532	949	830	≥20	0.5		
190726	None	N/A	N/A	N/A	N/A	N/A	DRB1*03:01/ 03:147	DRB1*07:01
	Lipoteichoic acid	1238	777	973	≥20	0.2		
	Borrelia burgdorferi	1723	1033	1103	≥20	0.2		
191016	None	4343	3111	2285	≥20	0	DRB1*01:01	DRB1*04:01
	Lipoteichoic acid	4216	2928	2141	≥20	0.1		
	Borrelia burgdorferi	4138	2999	2153	≥20	0.1		
191114	None	949	647	754	≥20	0.8	DRB1*01:01	DRB1*16:01
	Lipoteichoic acid	1614	1116	980	≥20	0.1		
	Borrelia burgdorferi	2302	1595	1262	≥20	0.1		
191202	None	221	156	195	≥20	0.9	DRB1*11:01	DRB1*15:01
	Lipoteichoic acid	552	393	468	≥20	0		
	Borrelia burgdorferi	659	476	490	≥20	0.5		
200212	None	1651	1109	1532	≥20	1	DRB1*11:03	DRB1*13:01
	Lipoteichoic acid	418	179	241	≥20	2.6		
	Borrelia burgdorferi	1025	520	530	≥20	0.3		
200218	None	3645	2341	1969	≥20	0.2	DRB1*01:01	DRB1*15:01
	Lipoteichoic acid	4302	2696	2064	≥20	0.3		
	Borrelia burgdorferi	4009	2647	1900	≥20	0.3		

(PE) Buffer and pelleted at 1,200 rpm at room temperature for 3 min. Cells were stained with CD14-PE (BD Pharmingen), CD11c-PerCP (BioLegend), HLA-DR-BV510 (BD Horizon), and Blue Fluorescent Reactive Dye (Life Technologies) for 15 min in BV Buffer (BD Horizon). At the end of the incubation period, cells were washed twice in PBS and resuspended in 100 μL PBS for flow cytometry analysis in a BD FACSAria II flow cytometer (BD Biosciences). All data was gated on $CD14^{-}$/$CD11c^{+}$ monocyte derived dendritic cells.

In vitro Activation of Monocyte-Derived Dendritic Cells for Immunopeptidome Isolation

Immature mo-DCs were seeded in T-25 cell culture flasks at a density of 2–15 × 10^6 cells in 7.5 mL of RPMI 1640 and 10% fetal bovine serum, supplemented with 2 mM L-glutamine and 20 μM ß-mercaptoethanol. Immature mo-DCs were stimulated with 1 μg/mL purified LTA or live *B. burgdorferi* strains A3 or B31-5A19 at a multiplicity of infection of 10 bacteria per cell for 24 h. Cells were incubated at 37°C, 5% CO_2, and >95% humidity. At the end of the incubation period, naturally processed and presented peptides were isolated from HLA-DR molecules by immunoprecipitation using a natural antigen processing assay (13). Briefly, cells were harvested and pelleted at 1,200 rpm for 10 min at 4°C. Culture flasks were washed with cold PE buffer, adherent cells were lifted, and this solution was used to pellet cells. Cells were resuspended in 400 μL/2 × 10^6 cells in lysis buffer (1% CHAPS, pepstatin, leupeptin, chymostatin, antipain, PMSF, and EDTA) and lysed for 1 h at 4°C in a rocking table. Cellular debris was removed by centrifugation at 10,000 RCF for 15 min to clear supernatant twice. Twenty μg of the anti-HLA-DR antibody L243 (purified from ATCC HB-55 hybridoma) was added to each sample and incubated overnight at 4°C. One hundred μL of Rec-Protein G-Sepharose 4B conjugate (Thermo Fisher Scientific) slurry was added to the antigen-antibody complex and incubated with gentle mixing for 2 h at room temperature. The agarose-antibody-antigen complex was washed with 500 μL of a 20 mM Tris, pH 7.4 and 150 mM NaCl solution. Peptides were eluted from the bead-antibody complex in 100 μL of 1% trifluoroacetic acid (TFA) and isolated using C18 spin columns (Thermo Fisher Scientific) according to manufacturer's instructions.

Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS)

Samples were processed in Waters Oasis MAX (Mixed-mode Anion eXchange) 96-well microelution plates using a Waters Positive Pressure-96 Processor prior to mass spectrometry analysis. Each Oasis well was conditioned with 100 μL of 4% H_3PO_4, samples were diluted 1:1 with 4% H_3PO_4, and transferred to the microelution plate. Pressure was applied to concentrate the peptides on the MAX phase, washed twice with 50 μl of 5% NH_4OH followed by two 50 μL washes of 20% acetonitrile. The flow through was discarded and a new clean 96-well plate was placed under the Oasis MAX plate. Peptides were eluted from the MAX phase with two 50 μL aliquots of 75% acetonitrile containing 1% TFA. Eluted peptides were dried down by speed vacuum centrifugation and reconstituted in 2% acetonitrile, 0.1% formic acid. Peptides were injected into a trap column, eluted over a 90 min, 2–90% acetonitrile gradient containing 0.1% formic acid at 300 nL per minute. Columns were packed in house using a New Objective 75 μm ID PicoFrit with ReproSil-Pur C18 AQ 3 μm stationary phase. Peptides were eluted with a Thermo Fisher Easy-nanoLC system interfaced with a Thermo Scientific Orbitrap Fusion Lumos Tribrid Mass Spectrometer. Peptides were analyzed with a data dependent 3 s cycle fragmentation method for the highest abundant precursors. Survey and MS2 scans were performed at 120,000 and 30,000 resolution, respectively. The mass spectrometry.raw files were searched with the PEAKS X software against the *Homo sapiens* RefSeq database protein sequences with no enzyme designation and allowing for variable modification of methionine oxidation and asparagine or glutamine deamidation. The resulting peptide identifications were filtered at a −10lgP-value of 20 using the PEAKS decoy-fusion algorithm.

Data Analysis

For flow cytometry analysis, statistical significance was calculated by comparing the 0 h unstimulated median MFI (Median Fluorescence Intensity) vs. median MFIs from each condition using the One-way ANOVA ($p < 0.0001$) statistical test followed by Dunnett's multiple comparisons test ($^{****}p < 0.0001$) using GraphPad Prism version 8.4.2.

The BioVenn web application (https://www.biovenn.nl) was used to generate area-proportional Venn diagrams to visualize overlap of all identified parent proteins using the SVG Only display option with the print numbers option selected and both the absolute nrs and percentages options selected as well (14). Corresponding ID lists of proteins found in common between all stimuli (Unstimulated, LTA, and *B. burgdorferi*) and those uniquely found in only one stimulus (Unstimulated or LTA or *B. burgdorferi*) were exported from the BioVenn web application using the Current Image Statistics lists.

The Advanced Biomedical Computing Center's biological DataBase network (bioDBnet, https://biodbnet-abcc.ncifcrf.gov/db/db2db.php) db2db Database to Database Conversions version 2.1 was used to convert non-redundant RefSeq Protein Accession numbers (input) exported from BioVenn's Current Image Statistics lists into UniProt Accession identifiers (output) (15).

The Laboratory of Human Retrovirology and Immunoinformatics's Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 Functional Annotation Clustering tool was used to cluster genes into Gene Ontology (GO) terms (16, 17). Individual ID lists of Uniprot Accession numbers were individually uploaded to DAVID (Step 1) under the Uniprot_Accession Identifier (Step 2) as a Gene List (Step 3). DAVID's default settings were unchecked in order to individually select GO terms BP_ALL (BP, Biological Processes) and CC_ALL (CC, Cellular Compartments) and the Functional Annotation Clustering tool was selected. Binning of all gene sets was rerun at a high classification stringency and an EASE score of 0.1 as the Enrichment Threshold. All other options were left at default settings. DAVID's algorithm assigns Enrichment Scores to each Annotation Cluster by calculating the geometric mean in -log scale of the cluster members' *p*-values. The *p*-values assigned to each GO term in the Annotation Clusters equals the Fisher Exact/EASE Score assigned to each GO term. Kappa statistics and fuzzy heuristic clustering measure the degree of common genes between two terms and groups similar terms according to kappa values (16, 17). Listings of enriched clusters were downloaded from DAVID's web application and bar graphs displaying the top 20 enrichment clusters (if DAVID thresholds were met) and corresponding *p*-values were graphed using GraphPad Prism version 8.4.2.

Core sequences of identified integrin α-M precursor peptides were predicted with the Denmark Technical University (DTU) NetMHCIIpan-3.2 server using default settings (18). Predicted core sequences were then submitted to DTU's Seq2Logo web-based sequence logo generation method (19). Kullback-Leibler type logos for specific *HLA-DRB1* alleles were generated using the Hobohm1 clustering method with a 0.63 clustering threshold, 200 pseudo counts, and 50 stacks per line. Amino acid coloring scheme was set to Seq2Logo defaults.

RESULTS

Borrelia burgdorferi Induces the Upregulation of Cell Surface HLA-DR

Dendritic cells play a key role in the initiation of the human adaptive immune response against invading pathogens. Engagement of the T cell receptor by MHC class II HLA-DR molecules loaded with a foreign antigen peptide on DCs is an essential signal necessary to initiate T cell activation (12). Accordingly, we investigated changes in cell surface expression of HLA-DR in mo-DCs at rest or upon stimulation with the Toll-like receptor-2 agonist LTA from *Staphylococcus aureus* or with live *B. burgdorferi* at baseline, 8, and 24 h post treatment. Our results showed that *B. burgdorferi* strains A3 and B31 at multiplicities of infection of 1 or 10, induce upregulation of HLA-DR on the surface of mo-DCs in a time and dose dependent manner when compared to unstimulated mo-DCs and those stimulated with LTA (**Figure 1**). These results indicate that *B.*

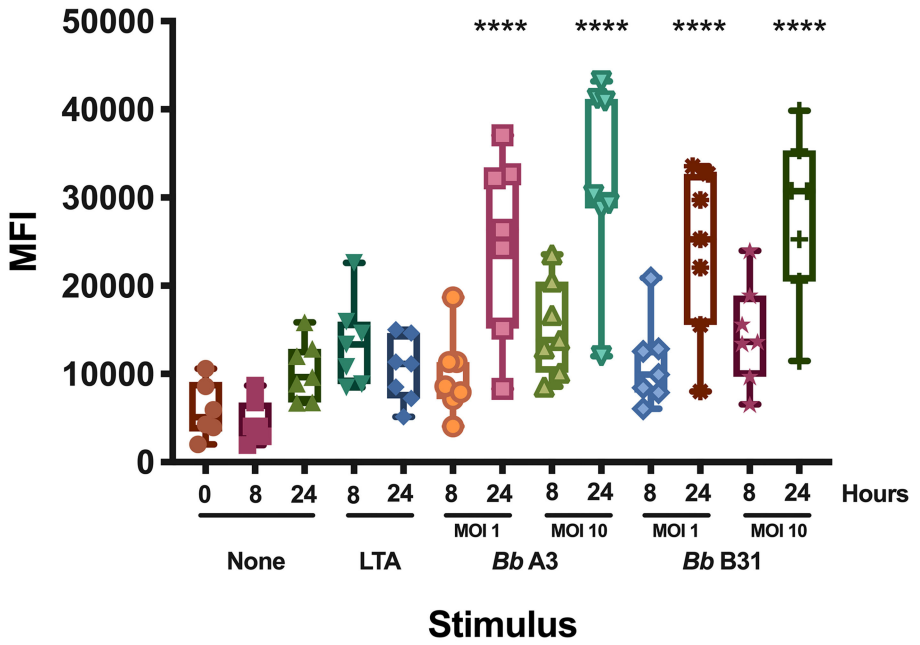

FIGURE 1 | Cell surface expression of HLA-DR in monocyte-derived dendritic cells. Expression of HLA-DR on mo-DCs left unstimulated or stimulated with LTA or live *B. burgdorferi* was measured at baseline, 8- and 24-h post incubation by flow cytometry. Statistical significance was calculated by comparing the 0 h unstimulated median MFI (Median Fluorescence Intensity) vs. median MFIs from each condition using the One-way ANOVA ($p < 0.0001$) statistical test followed by Dunnett's multiple comparisons test (****$p < 0.0001$) using GraphPad Prism version 8.4.2.

burgdorferi can supply the required signals to initiate dendritic cell maturation.

Parent Protein and Supporting Peptide Characteristics

We set out to define the mo-DC immunopeptidome after exposure to *Borrelia burgdorferi*. Monocyte-derived dendritic cells differentiated from PBMCs from 9 healthy donors (**Table 1**), were used as a source of APCs. The mo-DCs were either left unstimulated, stimulated with LTA or exposed to live *B. burgdorferi* for 24 h. The peptide-HLA-DR complexes were isolated by immunoprecipitation and peptides eluted from the HLA-DR groove were processed for LC-MS/MS identification. We set a stringent threshold for peptide identification in the PEAKS X software to −10lgP-values ≥ 20 (∼$p \leq 0.01$) in order to filter the identified human peptides at a 1% false discovery rate using the PEAKS decoy-fusion algorithm. Using this approach, we identified HLA-DR-associated peptides from every donor. The number of peptide-spectrum matches ranged from 221 to 4,343, with 156 to 2,999 peptide sequences detected (**Table 1**). We next identified the human parent proteins for all detected peptides. Across all subjects and conditions, we found 4,146 parent proteins as peptide sources in unstimulated mo-DCs, 4,070 parent proteins in mo-DCs stimulated with LTA, and 4,038 parent proteins in mo-DCs stimulated by *B. burgdorferi*. Peptides identified from self-proteins matched common characteristics of MHC class II presented peptides, with the average length of the most abundant peptides ranging between 9 and 26 amino acids (**Figure 2A**) and nested peptide sets at specific regions within the parent protein (**Figure 2B**). Using peptides derived from integrin α-M precursor, a peptide donor in all subjects, we generated amino acid binding motifs and sequence profiles using the Seq2Logo sequence logo generator. The constructed logo matched published MHC II binding motifs based on the individual's *HLA-DRB1* alleles (**Figure 2C**) (20). In general, peptides that bind class II MHC molecules share a hydrophobic residue at positions 1 and 9 [phenylalanine (F), tyrosine (Y), leucine (L), valine (V), isoleucine (I), alanine (A), glycine (G)], a negatively charged residue at position 4 [aspartic acid (D) or glutamic acid (E)], and an inclination for a basic residue at position 6 [lysine (K), arginine (N), histidine (H), glutamine (Q) or asparagine (N)] (21). Overall, these results validate the observed characteristics of the peptides identified in our assays as *bona fide* class II MHC-processed and presented epitopes.

Identification of Source Proteins for HLA-DR-Bound Peptides: Common and Unique Features Under Different Conditions

We next characterized the source proteins of identified HLA-DR bound peptides in all subjects and all conditions. The most commonly presented proteins in all stimuli were integrin α-M, vimentin, annexin A2, cathepsin B isoform 1 preprotein, HLA class II DR α chain precursor, hemoglobin subunit α, filamin-A, aminopeptidase N precursor, actin cytoplasmic, HLA class I A-1 α chain, HLA class I Cw-I α chain precursor,

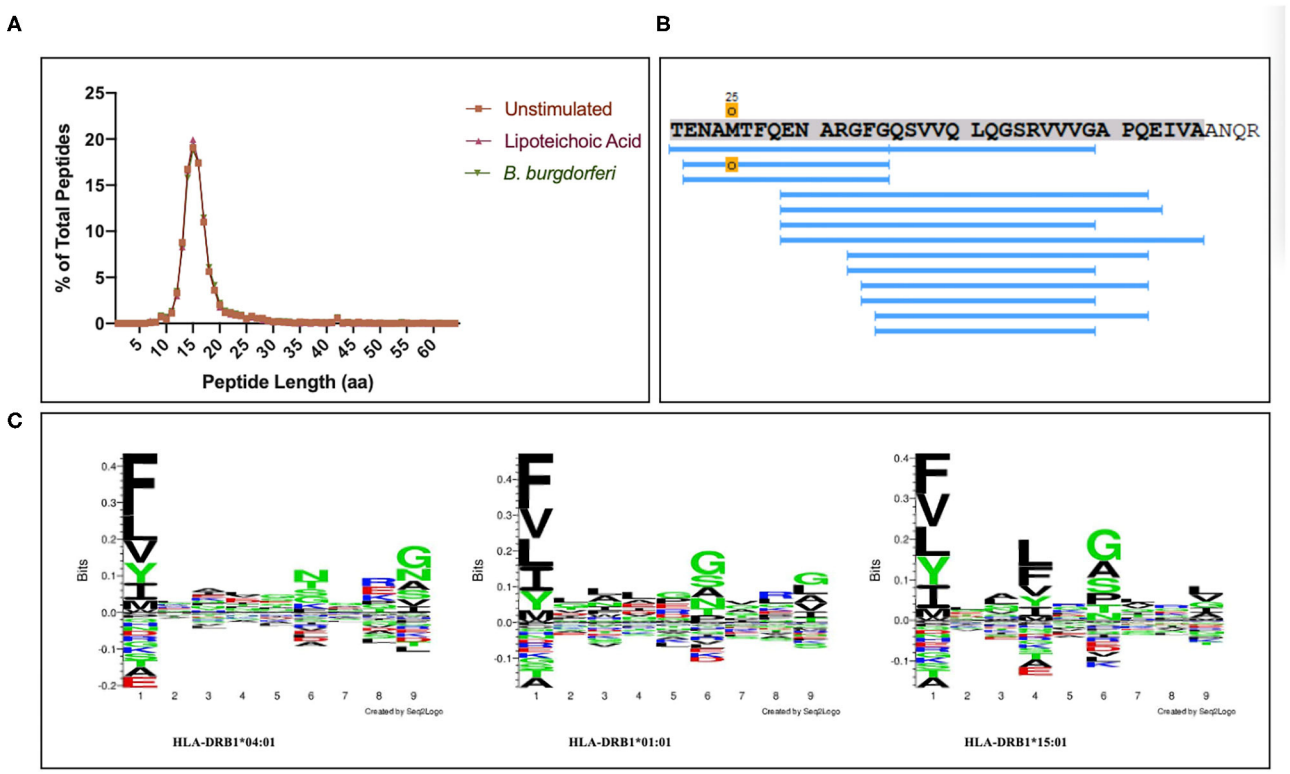

FIGURE 2 | Identified peptides meet characteristics of MHC class II processed and HLA-DR presented peptides. **(A)** Percent of all peptides identified by LC-MS/MS from mo-DCs left at rest or stimulated with LTA or live *B. burgdorferi* for 24 h, based on peptide length. GraphPad Prism version 8.4.2 was used for graphical representation. **(B)** Representative nested set of peptides derived from the protein integrin α-M precursor (residues 21–56, donor 191016) characteristic of MHC class II processed proteins. **(C)** Seq2Logos of peptides derived from integrin α-M precursor from donors 190529, 191016, and 200218 with *HLA-DRB1* alleles 04:01, 01:01, or 15:01. Seq2Logo default amino acid colors D and E red; N, Q, S, G, T, and Y green; R, K, and H blue; and unassigned amino acids black.

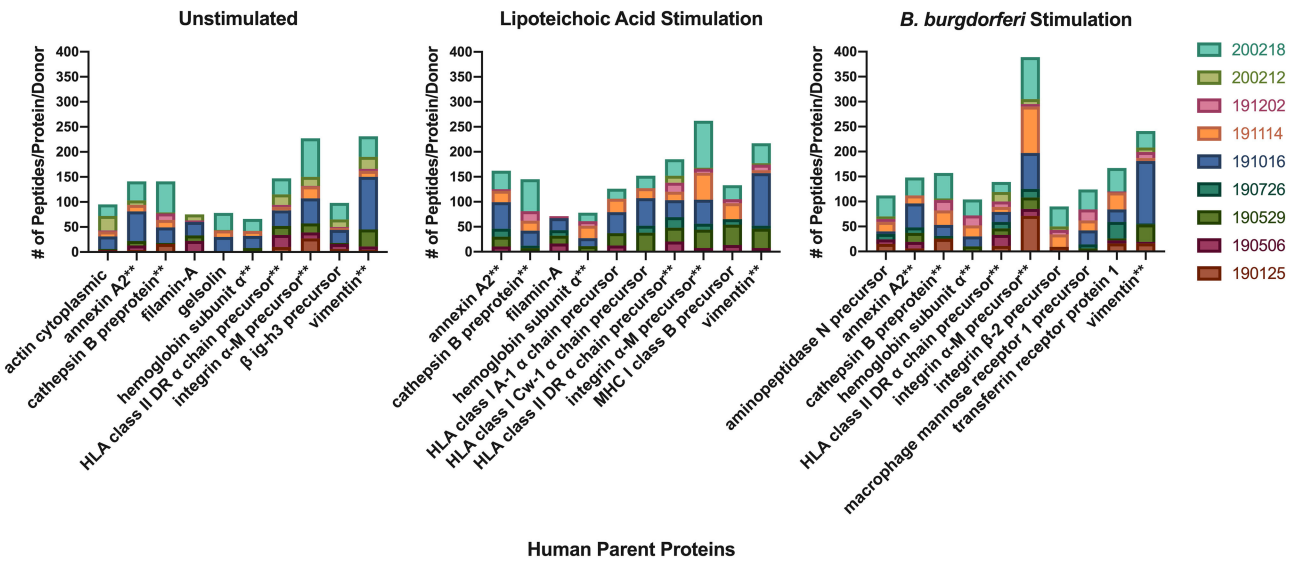

FIGURE 3 | Most abundantly presented parent proteins in all stimuli. Number of identified peptides from the top 10 parent proteins from healthy mo-DCs left at rest (left panel) or stimulated with LTA (middle panel) or live *B. burgdorferi* (right panel) for 24 h. GraphPad Prism version 8.4.2 was used for graphical representation. **Denotes parent proteins identified in all stimuli.

TABLE 2 | Most abundantly identified parent proteins presented by HLA-DR molecules detected in all donors and conditions.

Parent protein	Accession number	Stimulus	# of Donor Peptides								
			190125	190506	190529	190726	191016	191114	191202	200212	200218
Actin cytoplasmic 1 & 2	NP_001092.1 NP_001186883.1	None	3	1	2	–	25	10	2	29*	23
		LTA	–	1	6	3*	28*	12	5	–	28*
		Bb	4	–	7	–	34	20*	–	–	26
Aminopeptidase N precursor	NP_001141.2	None	3	4	–	–	9	3	5	8	39
		LTA	–	3	–	7	12	16	6	–	51
		Bb	15	9	–	11	5	19	6	5	42
Annexin A2 (isoform 1 & 2)	NP_001002858.1 NP_001002857.1	None	4	9	9	–	59	13	–	9	38
		LTA	–	10	20	16	53	23	3	–	37
		Bb	6	13	18	11	48	16	–	–	36
Cathepsin B isoform 1 preprotein	NP_001899.1	None	16	–	2	–	31	15	13	2	62
		LTA	–	–	7	5	30	20	19	–	64
		Bb	25	–	2	4	22	29	21	3	51
Filamin-A (isoform 1 & 2)	NP_001447.2 NP_001104026.1	None	–	22	11	–	27	–	3	12	–
		LTA	–	16	16	11	24	–	4	–	–
		Bb	2	26	15	10	24	–	6	1	–
Gelsolin (isoform a-g)	NP_000168.1	None	–	–	2	–	28	13	–	1	34
		LTA	–	–	2	–	28	32	–	–	28
		Bb	1	–	1	–	28	25	–	–	32
Hemoglobin subunit α	NP_000508.1	None	–	–	8	–	24	10	–	–	24
		LTA	–	–	11	–	16	25	9	–	17
		Bb	–	–	10	–	20	22	20	–	32
HLA class I A-1 α chain A*03:01:0:01 precursor	NP_002107.3	None	–	7*	18*	–	44*	7*	–	2*	16*
		LTA	–	12*	25*	–	42*	27*	–	–	20*
		Bb	6*	13*	21*	12*	48*	19*	3	–	12*
HLA class I Cw-1 α chain precursor	NP_002108.4	None	–	10*	25*	-	55*	8*	–	4	21*
		LTA	–	–	38*	14*	55*	20*	–	–	25*
		Bb	6*	14*	33*	10*	58*	14*	3	–	14*
HLA class II DR α chain precursor	NP_061984.2	None	10	24	18	–	31	7*	4	21	32
		LTA	–	20	28	21	34	17	18	14	33
		Bb	11	22	13	13	20	10	11	19	20
Integrin α-M (isoform 1 & 2)	NP_000623.2 NP_001139280.1	None	27*	12	18	–	50	24*	1	18	77*
		LTA	–	7	37	12*	48	54*	8	2	94
		Bb	71*	14	23	17*	72	93*	5	10	84
Macrophage mannose receptor 1 precursor	NP_002429.1	None	–	–	4	–	23	1	3	–	33
		LTA	–	–	7	–	15	5	12	–	36
		Bb	1	1	4	8	28	20	22	–	40
MHC class I B precursor	NP_005505.2	None	1	10*	26*	–	–	11*	–	4	25*
		LTA	–	13*	41*	11*	–	32*	8	–	28*
		Bb	7*	14*	32*	7*	–	25*	3	–	21*
Transferrin receptor protein (isoform 1–3)	NP_003225.2	None	4	–	–	–	9	–	–	1	11
		LTA	–	–	–	6	15	3	–	–	19
		Bb	16	6	4	33	25	34	2	–	47
TGF-ß-induced protein ig-H3	NP_000349.1	None	6	10	2	–	26	4	2	15	33
		LTA	–	7	4	9	21	10	1	–	21
		Bb	8	10	–	10	14	9	2	–	13
Vimentin	NP_003371.2	None	4	7	34	–	105	12	4	24	41
		LTA	–	7	39	6	105	6	11	3	40
		Bb	16	3	35	2	125	6	12	9	33

Denotes that self-peptides could be derived from multiple related isoforms.

major histocompatibility complex class I B precursor, transferrin receptor protein, macrophage mannose receptor 1 precursor, transforming growth factor beta-induced protein ig-H3, and gelsolin (**Figure 3** and **Table 2**). Peptides from all parent proteins that were commonly identified were found in at least 4 out of the 9 (44%) healthy subjects. Globally, we observed significant overlap of source proteins, with 2,460 proteins (36.7%) contributing peptides for presentation in all donors and all stimuli, while smaller numbers of proteins were shared between two stimulation conditions (**Figure 4**). Interestingly, 1,048 (15.6%), 730 (10.9%), and 1,020 (15.2%) of all identified source proteins were exclusively presented by immature mo-DCs, LTA-stimulated mo-DCs and *B. burgdorferi*-stimulated mo-DCs, respectively (**Figure 4**). Individual donors paralleled this trend regardless of HLA-DR genotype (**Supplementary Figure 1**). This latter observation indicates that monocyte-derived dendritic cells will display self-peptides derived from unique protein sources depending on their physiological state.

Overlapping Functional Annotation Clustering of Parent Proteins

We used DAVID's functional annotation tool to cluster parent proteins identified from all donors and all stimulation conditions into biologically similar terms (16, 17). Accordingly, unique RefSeq protein accession numbers for identified source proteins were converted into UniProt accession identifiers that were categorized into shared cellular compartments and biological processes (15). First, we analyzed those 2,460 proteins that were common peptide sources in all conditions (**Figure 4**). Conversion of these 2,460 protein identifiers to gene annotations added redundancy to the listing as a result of protein isoforms. Consequently, we removed all duplicate genes and analyzed 1,852 unique gene identifiers. Binning of gene sets into cellular compartments resulted in 18 functional annotation clusters (**Figure 5A**). Top clusters were enriched with genes found in extracellular vesicles or exosomes; cell-cell adherens or anchoring junctions; focal adhesions; cytoplasmic, membrane-bound vesicles; membrane regions, microdomains or rafts; and integral and intrinsic components of the lumenal side of the endoplasmic reticulum membrane. Similar settings were used for clustering of gene sets into biological processes, yielding 186 functional annotation clusters (**Figure 5B**). Top clusters in this enrichment analysis included antigen processing and presentation; interspecies interaction between organisms or multi-organism cellular processes; antigen processing and presentation of peptide or polysaccharide antigen via MHC class II, movement of cell or subcellular components; immune response-regulating signaling pathways; catabolic processes; and establishment of protein localization or protein transport. Notably, enriched cellular compartments reflect the biological processes commonly taking place inside the cell. For example, antigen processing and presentation of peptides, which takes place in cytoplasmic, membrane-bound vesicles and with molecules that are integral components of the lumenal side of the endoplasmic reticulum membrane, was the top biological process among all donors and all stimuli. These results strongly suggest that common biological processes in mo-DCs require extensive cellular membrane involvement from numerous compartments.

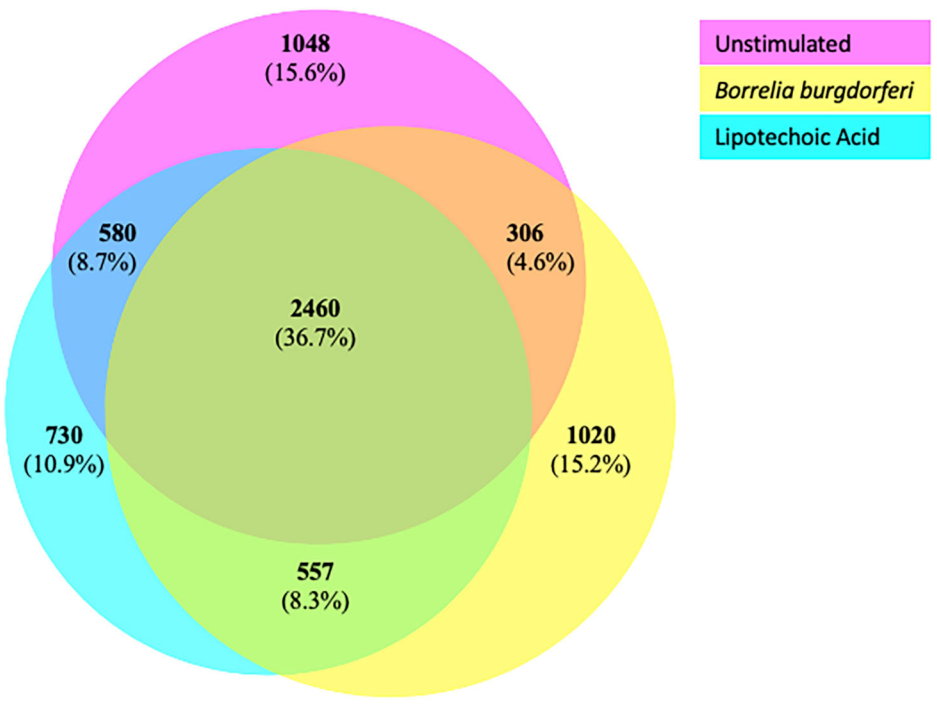

FIGURE 4 | Common and exclusive parent proteins presented in all stimuli. BioVenn diagram illustrating overlap and exclusivity in source proteins identified by LC-MS/MS from mo-DCs left at rest (pink circle) or stimulated with LTA (aqua circle) or live *B. burgdorferi* (yellow circle) for 24 h.

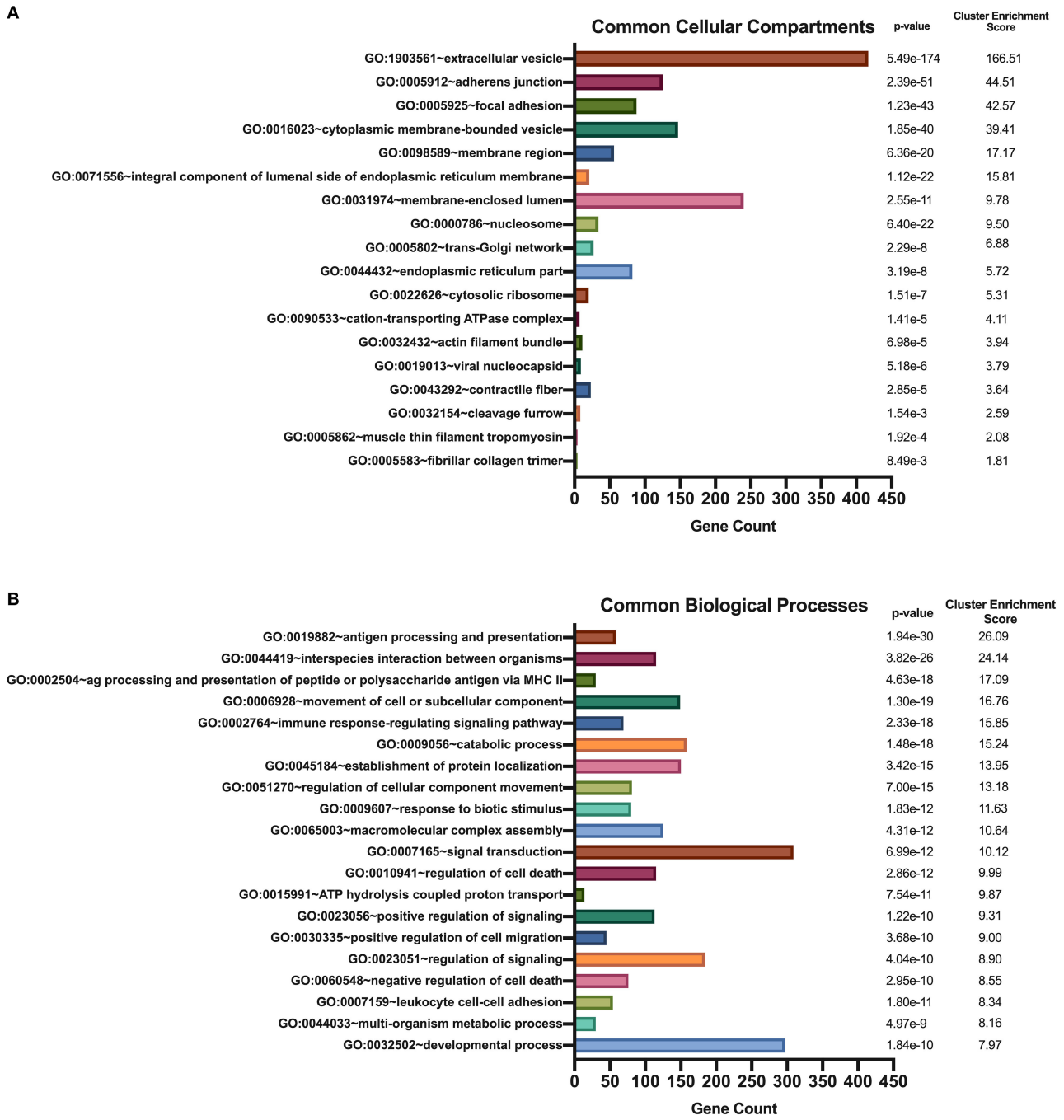

FIGURE 5 | Enriched Gene Ontology terms derived from source proteins identified in all stimuli. DAVID functional annotation clustering of cellular compartments **(A)** and biological processes **(B)** identified in all healthy donors' mo-DCs that were left at rest or stimulated with LTA or live *B. burgdorferi* for 24 h. RefSeq Protein Accession numbers were converted to UniProt gene identifiers for DAVID compatibility. GraphPad Prism version 8.4.2 was used for graphical representation.

Common and Unique Functional Annotation Clustering of Parent Proteins Under Different Conditions

We hypothesized that exposure of mo-DCs to *B. burgdorferi* will alter processing and presentation by HLA-DR molecules in dendritic cells, eliciting changes in the immunopeptidome by triggering distinct biological processes and involving cellular compartments exclusive to the cellular response to *B. burgdorferi*. We used DAVID's functional annotation tool to identify cellular compartments and biological processes exclusive to unstimulated cells, LTA-stimulated mo-DCs or live *B. burgdorferi*-stimulated mo-DCs (**Figure 6**). Conversion of RefSeq Protein Accession numbers to UniProt gene annotations reduced the number

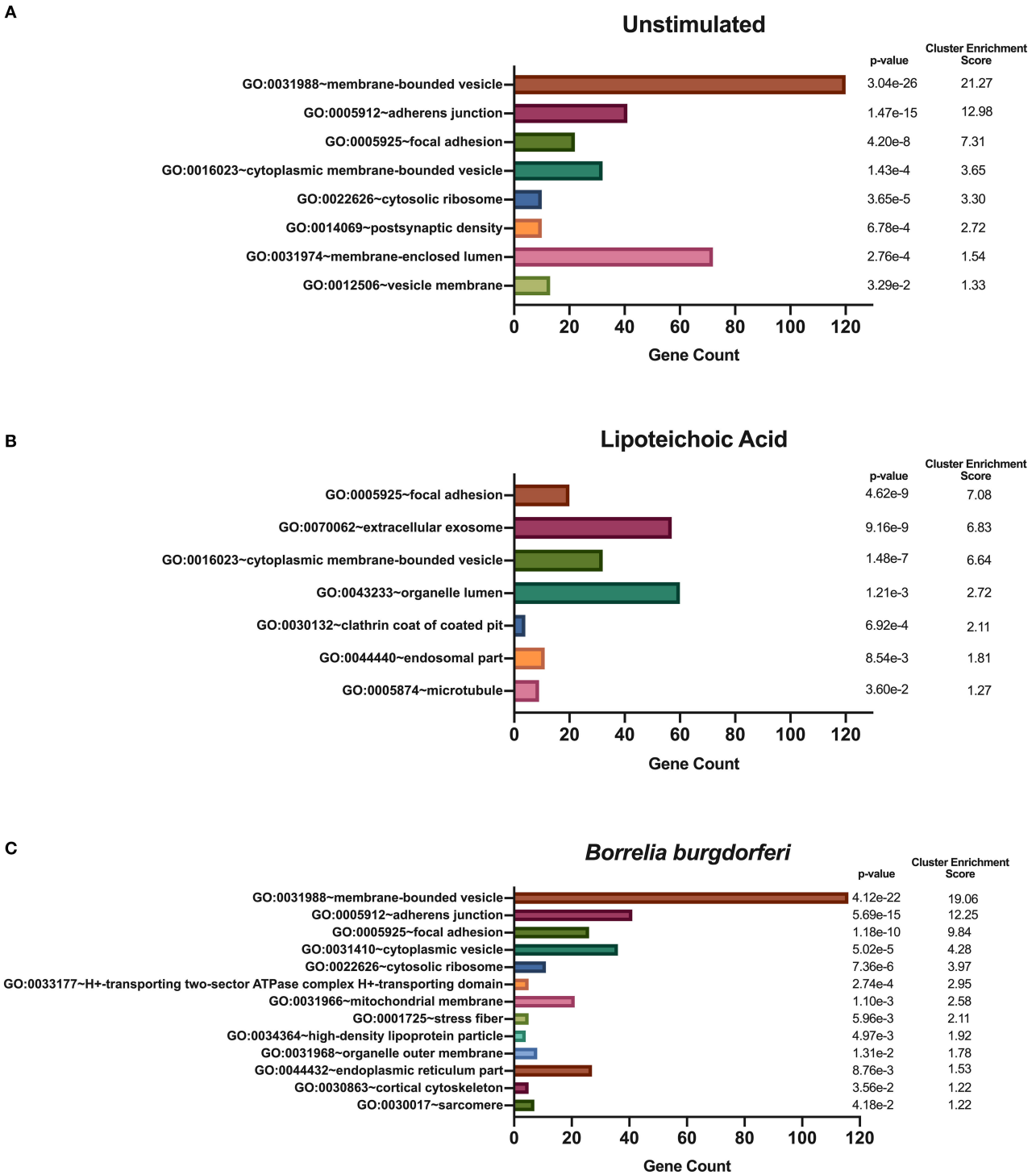

FIGURE 6 | Enriched cellular compartments derived from source proteins identified exclusively in each individual stimulus. DAVID functional annotation clustering of cellular compartments identified in mo-DCs that were left at rest **(A)** or stimulated with LTA **(B)** or live *B. burgdorferi* **(C)** for 24 h. RefSeq Protein Accession numbers were converted to UniProt gene identifiers for DAVID compatibility. GraphPad Prism version 8.4.2 was used for graphical representation.

of identified source proteins from 1,048 to 672 genes in unstimulated mo-DCs, from 730 proteins to 435 genes in LTA-stimulated cells, and from 1,020 proteins to 653 genes in *B. burgdorferi*-stimulated mo-DCs. The most significant functional annotation clusters enriched in LTA- and live *B. burgdorferi*-stimulated mo-DCs overlap with those found in all donors

regardless of stimuli. These clusters include membrane-bound or extracellular vesicles or exosomes; adherens or anchoring junctions; focal adhesions; and cytoplasmic, membrane-bounded vesicles (**Figure 6**). Interestingly, the genes enriched in these clusters differ from those identified in unstimulated mo-DCs. Furthermore, in LTA-stimulated mo-DCs involvement of clathrin coat of coated pits or clathrin-coated endocytic vesicles; the endosomal or vacuolar membrane; and microtubules were uniquely enriched (**Figure 6B**). On the other hand, *B. burgdorferi* stimulation elicited involvement of other cellular compartments such as the proton-transporting two-sector ATPase complex; the mitochondrial membrane; high-density lipoprotein or plasma lipoprotein particles; organelle or mitochondrial outer membrane; and the cell cortical cytoskeleton (**Figure 6C**). These results suggest that *B. burgdorferi* stimulation of dendritic cells leads to utilization and subsequent sampling of unique compartments within the cell that become major sources of self-peptides.

We used DAVID's functional annotation tool to identify unique biological processes in LTA- and in *B. burgdorferi*-stimulated mo-DCs (**Figure 7**). Consistent with previous results, several gene ontology terms in the top annotation clusters identified in both conditions overlap with those enriched in the all donors and all conditions set. However, the genes comprising these clusters differ from those observed in the all donors and all stimuli grouping. Biological processes unique to LTA-stimulated mo-DCs include intracellular protein transport; positive regulation of the immune response; phosphate-containing compound metabolic processes; cellular response to indole-3-methanol; and nucleobase-containing compound transport. Interestingly, enriched clusters unique to *B. burgdorferi*-stimulated mo-DCs include ion transmembrane transport; ribosome biogenesis; ncRNA processing; ncRNA metabolic processes; response to lipopolysaccharide; organophosphate biosynthetic processes; ribonucleotide metabolic processes, and muscle hypertrophy and adaptation. These results lend additional support that *B. burgdorferi* stimulation of DCs leads to utilization and subsequent sampling of unique cellular compartments and pathways for processing and presentation on MHC class II molecules.

Autoantigens in Lyme Arthritis

Previous studies identified peptides from annexin A2, apolipoprotein B-100, thymidine phosphorylase (endothelial cell growth factor), and stromelysin-2 (matrix metalloproteinase 10) as targets of autoreactive T cells in a subset of human Lyme disease patients (22–24). We interrogated our data to determine whether these proteins were sources of HLA-DR-binding peptides and identified peptides from annexin A2, apolipoprotein B-100 and thymidine phosphorylase. Our analysis showed that all donors presented peptides from annexin A2, with five out of nine donors presenting peptides regardless of stimulus or HLA-DR genotype (**Supplementary Figure 2**). We identified 107 unique (498 total) annexin A2 peptides, including nested peptide sets containing the predicted promiscuous HLA-DR-binding sequences ^{50}GVDEVTIVNILTNRSNAQR^{68} and ^{164}SGDFRKLMVALAKGRRA^{180}, along with peptide clusters from other regions within the protein (**Supplementary Figure 2**). Our analysis did not identify a previously reported sequence (^{285}DKVLIRIMVSRSEVD^{299}) found to be T cell reactive in patients with Lyme arthritis (24).

We also detected peptides from apolipoprotein B-100 in seven out of nine donors. Interestingly five donors (donors 190506, 190726, 191114, 191202, and 200218), expressing *HLA-DRB1* alleles 03:01/03:02, 03:01/07:01, 01:01/16:01, 11:01/15:01, and 01:01/15:01 respectively, presented peptides from this protein only when stimulated with LTA or live *B. burgdorferi*. We found 22 unique (58 total) apolipoprotein B-100 peptides. Included were nested sets of the peptide ^{655}IEGNLIFDPNNYLPK^{669}, which was previously identified in the synovial tissue in a patient with antibiotic-refractory Lyme arthritis (*HLA-DRB1**03:01/03:05 alleles) (23), and peptides from other regions within the protein (**Figures 8A–C**).

Lastly, four out of nine donors (donors 190506, 190726, 191016, and 200218), expressing *HLA-DRB1* alleles 03:01/03:02, 03;01/07:01, 01:01/04:01, and 01:01/15:01 respectively, presented 8 unique (23 total) peptides from thymidine phosphorylase. The nested sets of peptides included the previously predicted HLA-DR-binding sequence A^{52}DIRGFVAAVVNSAQGAQI^{71} and the peptide L^{340}GRFERMLAAQGVDPG^{355} (**Figure 9A**), which was previously identified in a patient with antibiotic-refractory Lyme arthritis (*HLA-DRB1**01:01 alleles) (22). Notably, we did not find the predicted S^{220}KKLVEGLSALVVDV^{234} peptide, but did identify a smaller overlapping sequence (**Figures 9B,C**).

DISCUSSION

The observation that *B. burgdorferi*-induced changes led to robust expression of HLA-DR on the surface of monocyte-derived dendritic cells in a time- and dose-dependent manner led us to reason that the HLA-DR associated self-immunopeptidome was altered, and formed the premise for this study. Using the natural processing and presentation capabilities of mo-DCs, we isolated sufficient peptide-HLA-DR complexes (13) to define the self-immunopeptidome at steady state, and its variation during stimulation with the TLR-2 ligand lipoteichoic acid or with live *B. burgdorferi*. In all conditions, we isolated peptides that varied in length (seven to 65 amino acids long) (25) averaging 15 residues, the optimal length of MHC class II presented antigens. Commonly presented peptides were found in nested sets, with their predicted core matching binding motifs associated with the donors' *HLA-DRB1* expressed alleles (20). Isolated peptides were mostly derived from proteins associated with membrane bound compartments, including the MHC II compartment and its cargo, the cytoskeleton, and proteins involved in cell adhesion and migration (**Figure 3**). Overall, the isolated peptides exhibited bona fide characteristics attributed to MHC Class II processing. Therefore, we believe that the class II processing and presentation pathway was fully functional in mo-DCs under the conditions studied.

The top biological process in mo-DCs irrespective of stimulus was antigen processing and presentation, clearly highlighting the importance of this process in the function of mo-DCs. Genes encoding proteins enriched in this GO

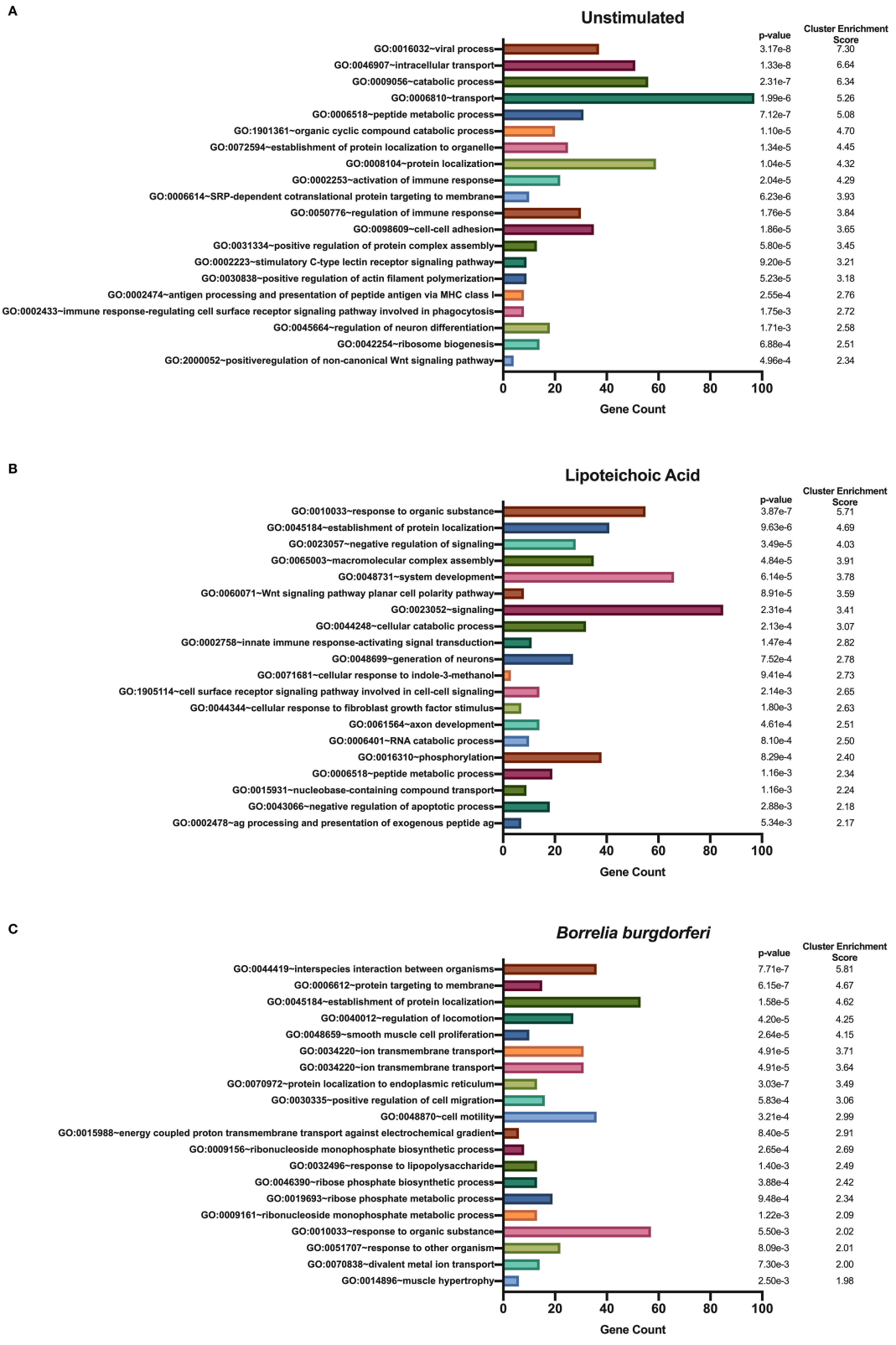

FIGURE 7 | Enriched biological processes derived from source proteins identified exclusively in each individual stimulus. DAVID functional annotation clustering of biological processes identified in mo-DCs that were left at rest **(A)** or stimulated with LTA **(B)** or live *B. burgdorferi* **(C)** for 24 h. RefSeq Protein Accession numbers were converted to UniProt gene identifiers for DAVID compatibility. GraphPad Prism version 8.4.2 was used for graphical representation.

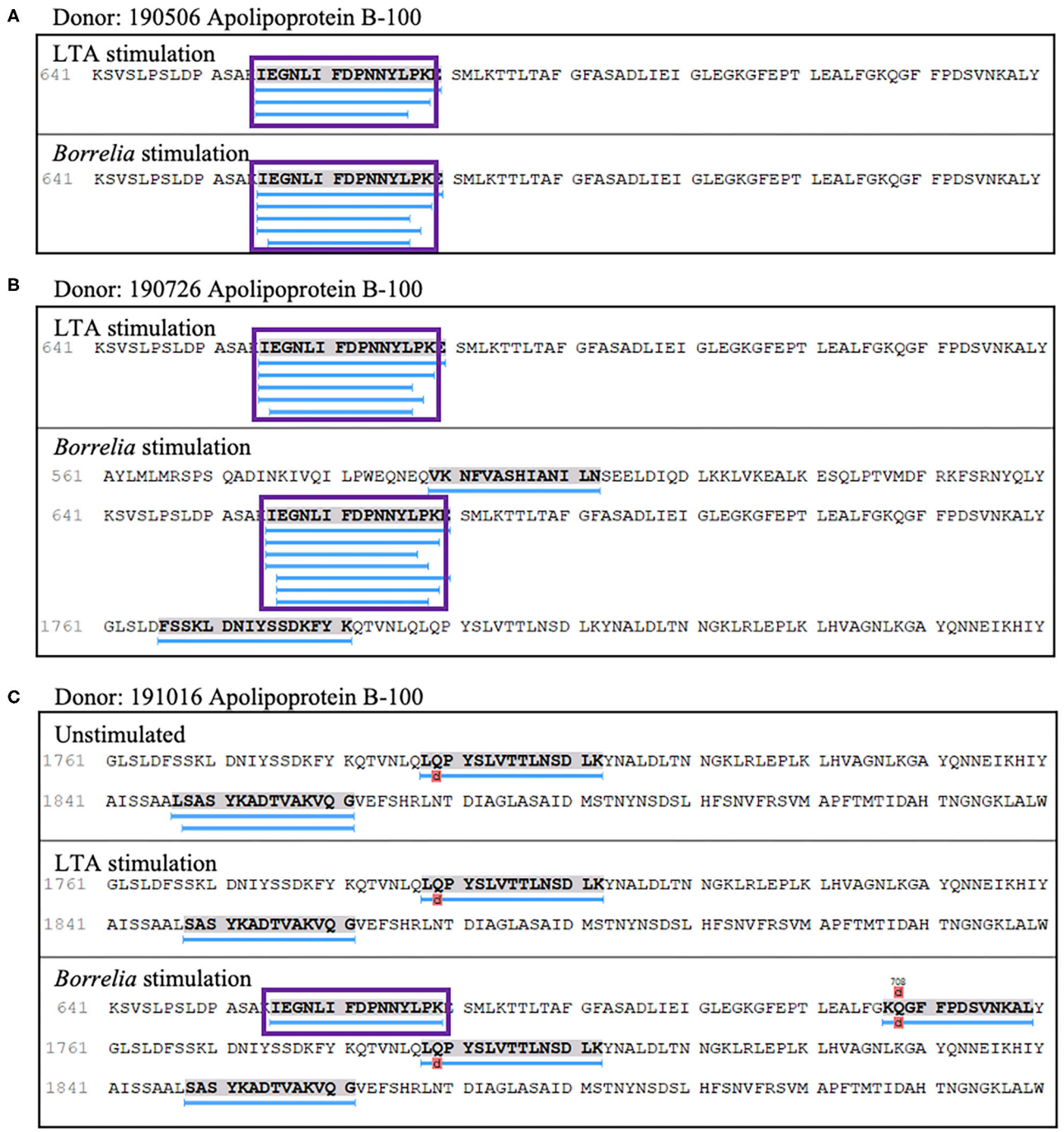

FIGURE 8 | Apolipoprotein B-100 presentation is associated with LTA and *Borrelia* stimulation. **(A)** Representative region of apolipoprotein B-100 previously reported as an autoantigenic epitope (purple rectangle) detected in mo-DCs from healthy donor 190506 expressing HLA-DRB1*03:01/03:02 stimulated with LTA (top panel) or *B. burgdorferi* (bottom panel) and healthy donor 190726 expressing HLA-DRB1*03:01/07:01 **(B)**. **(C)** Representative autoantigenic epitope of apolipoprotein B-100 detected in mo-DCs from healthy donor 191016 expressing HLA-DRB1*01:01/04:01 stimulated with *B. burgdorferi* (bottom panel).

term include pathogen recognition molecules such as *cd1b* (P29016), *cd1c* (P29017), and *cd209* (Q9NNX6), cathepsins (*ctsd* (P07229), *ctsh* (P09668), *ctsl* (P07711), and *ctss* (P25774), *cd74* (P04233), major histocompatibility complex, class II (*hla-dm*, *hla-do*, *hla-dp*, *hla-dq*, *hla-dr*) and class I molecules (*hla-a* (P04439), *hla-b* (P01889), *hla-c* (P10321), *hla-e* (P13747), *hla-f* (P30511), *hla-g* (P17693), and proteasome subunits (*psmd1* (Q99460), *psma3* (Q43242), *psma7* (P51665), *psmb1*(P20618) among others (**Supplementary List 1**). Interspecies interaction between organisms and antigen processing and presentation

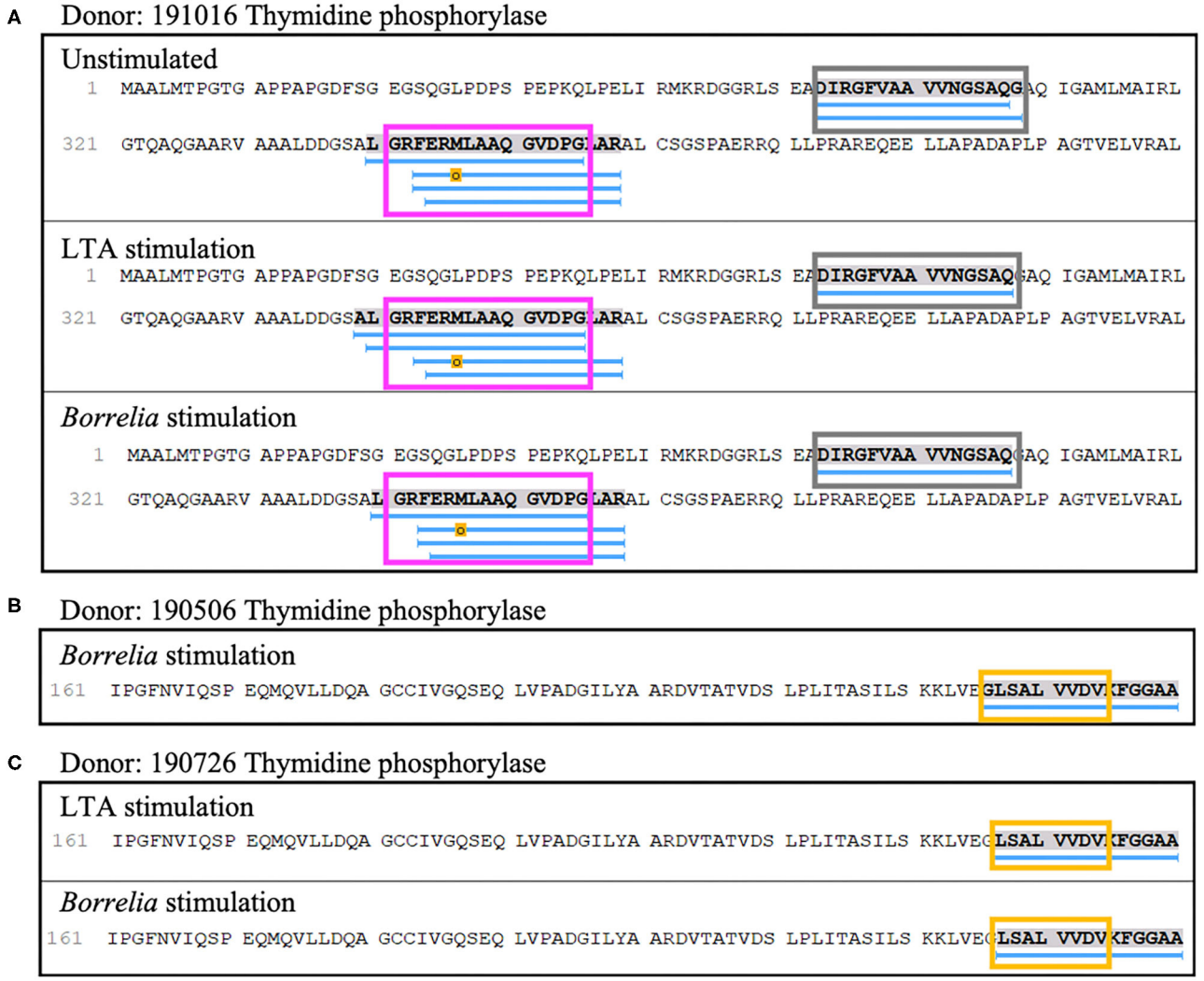

FIGURE 9 | Thymidine phosphorylase presentation is associated with LTA and *Borrelia* stimulation. **(A)** Representative region of thymidine phosphorylase previously reported as an autoantigenic epitope (pink rectangle) identified in mo-DCs at rest (top panel), stimulated with LTA (middle panel) or *B. burgdorferi* (bottom panel) from healthy donor 191016 expressing HLA-DRB1*01:01/04:01. A previously predicted promiscuous binding sequence (gray rectangle) was also detected in this donor. **(B)** Partial representative region of thymidine phosphorylase previously reported as an autoantigenic epitope (yellow rectangle) identified in mo-DCs stimulated with *B. burgdorferi* from healthy donor 190506 expressing HLA-DRB1*03:01/03:02. **(C)** Partial autoantigenic epitope was also detected in healthy donor 190726 expressing HLA-DRB1*03:01/07:01.

of peptide or polysaccharide antigen *via* MHC class II were also top clusters in shared biological processes between mo-DCs regardless of stimulus, again emphasizing the significance of these processes during both steady state and pathogenic challenge.

Interestingly, the immunopeptidome was significantly altered following stimulation with whole viable *B. burgdorferi*. This was evident not only in the identified peptides, but also in the source proteins that clustered to specific biological processes. Accordingly, the most significant biological process in *B. burgdorferi*-stimulated mo-DCs involved proteins that participate in interspecies interaction between organisms. These proteins are absent from the cluster in the shared biological processes, thus are unique to *Borrelia*-stimulated mo-DCs, and have been implicated in the immune response against pathogens, including *ccr5* (26) (P51681), *stat3* (P40763), *tnfaip3* (27) (P21580), *icam1* (P05362), and *cd86* (P42081) among others (**Supplementary List 2**). Identification of these peptides as part of the HLA-DR immunopeptidome from *Borrelia*-stimulated mo-DCs provides insights into novel biological process relevant to human Lyme disease. For example, potential involvement of the C-C motif chemokine receptor 5 (CCR5) in the interaction of *B. burgdorferi* and mo-DCs provides a novel pathway for early cytokine production during challenge with the *Borrelia* spirochete. Notably, engagement of CCR5 by the HIV-1 glycoprotein gp120 leads to increased production

of interleukin-6 (IL-6), resulting in dysregulation of STAT3 signaling and subsequent impairment of DC functionality (28). In addition, aberrant TNFAIP3 signaling also leads to increased IL-6 production in dendritic cells, implicating TNFAIP3 in the development of autoimmunity in dendritic cells as well as B-cells (27). IL-6 expression has been shown to be upregulated in patients with acute Lyme disease and remain elevated months after antibiotic treatment (29), suggesting that increased IL-6 levels in Lyme patients may play a role in the development of chronic symptoms. Thus, future studies aimed at elucidating the roles of IL-6, CCR5, STAT3, and TNFAIP3, potential cross-talk between these signaling pathways and how these may affect functionality of dendritic cells during *B. burgdorferi* challenge should be explored.

The top biological process in LTA-stimulated mo-DCs was response to organic substances. Genes associated with this GO term include *cd63* (P08962) and *mapkapk2* (P49137). CD63 is a cell surface-associated receptor that can be found in endosomes internalized into the cell *via* clathrin-dependent endocytosis (30), the known route of internalization of LTA (31). MAPKAPK2, a pro-inflammatory effector kinase, has been implicated in the innate immune response (32), signaling downstream of p38α, thus offering a differential response than that undertaken after *B. burgdorferi* stimulation. Further, viral process was the most significant biological process in mo-DCs at steady state, with none of the above-mentioned genes represented in this GO term. Other genes such as *cd46* (complement inhibitor; P15529) (33), *il10rb* (Q08334) (34), *ifnar2* (P48551), and *tgfb1*(P01137) that code for proteins implicated in viral immunity were enriched in this GO term, suggesting that antiviral processes are a main focus for mo-DCs at rest. Collectively, these findings reveal that the HLA-DR-bound self-immunopeptidome presented by mo-DCs is dynamic in nature and changes with the activation state of the cell reflecting differential functional capabilities. These studies will form the basis for future work investigating how *B. burgdorferi* impacts the function of mo-DCs, a cell type known to drive early innate and adaptive immune processes that inarguably impact the ability of the host to control infection.

Genetic susceptibility to autoimmune diseases is strongly associated with specific HLA alleles (35). A significant number of individuals with untreated Lyme disease will develop Lyme arthritis, a condition largely responsive to antibiotic treatment, but a subset of patients suffer from long term inflammation or antibiotic-refractory Lyme arthritis. In antibiotic-refractory Lyme arthritis, a late manifestation of *B. burgdorferi* infection and a condition with autoimmune components, the *HLA-DRB1* alleles 01:01, 04:01, and 15:01 are genetically linked to disease pathogenesis (36). Yet, presentation of autoantigenic peptides in Lyme arthritis by donors in our healthy cohort with risk HLA alleles, suggests that it is unlikely these alleles alone are responsible for the onset of disease. Rather, a breakdown in tolerance to self-antigens, triggered by infection with *B. burgdorferi*, may contribute to the development of dysregulated immunity and ultimately Lyme arthritis. Relevant to this study is that several proteins have been identified as targets of self-reactive $CD4^+$ T cells and autoantibodies in Lyme arthritis. These proteins were also identified using a mass spectrometry based approach, identifying HLA-DR associated peptides uniquely expressed in inflamed synovial tissue from patients with Lyme arthritis (22–24). Interestingly, we isolated peptides derived from three of these proteins: annexin A2, apolipoprotein B-100 and thymidine phosphorylase (endothelial cell growth factor).

Annexin A2, a Ca^{2+}-regulated membrane binding protein with key roles in membrane-cytoskeleton and membrane-membrane binding events, has been shown to be an autoantigen in several immune-mediated diseases including anti-phospholipid syndrome and rheumatoid arthritis (RA) (24). We found that annexin A2 was a major peptide donor in all subjects under all conditions. Among the peptides we isolated were two sequences identified by others as T cell targets and shown to be promiscuous HLA-DR binders (24). This feature is consistent with our finding that these peptides were isolated in all our subjects regardless of *HLA-DR* allele. A previous model was presented to explain how annexin A2 may become immunogenic and contribute to autoimmunity (24). In this model the "first hit" was driven by the presence of the spirochete, which facilitated annexin A2 uptake, processing, and presentation. Our results identifying auto-immunogenic annexin A2 peptides bound to HLA-DR in unstimulated mo-DCs implies that this first hit is spirochete independent. We hypothesize that mo-DCs, which appear to be constitutively processing and presenting annexin A2 peptides, are driving tolerance in a low-level self-reactive T cell population that is well-regulated, likely in the secondary lymphoid tissue. These cells can then be activated, as previously proposed (2nd hit), when target tissues upregulate autoantigen expression in an inflammatory setting, allowing local APCs to drive this self-reactive T cell population to expand and differentiate into effector cells. As noted previously, annexin A2 is upregulated in the inflamed joints of Lyme arthritis patients (24).

Peptides derived from thymidine phosphorylase (endothelial cell growth factor) were also identified in four of nine subjects in our study. Among the peptides isolated are two previously identified autoreactive, promiscuous binders in subjects expressing *HLA-DRB1* alleles 03:01/03:02 and 03:01/07:01, which are not considered risk alleles in Lyme arthritis (22). Notably, we did not identify the two non-promiscuous *HLA-DRB1**01:01 and 04:01 binders in subjects expressing those alleles, irrespective of the presence of live *Borrelia*. This finding suggests that these peptides may become immunogenic only in inflamed tissue. It is unknown whether thymidine phosphorylase is expressed by mo-DCs, yet its expression is upregulated in many malignancies, as well as during tissue regeneration and repair where it functions as a potent angiogenesis factor (37). Also, expression of thymidine phosphorylase is uniquely present in the synovial fluid and synovial tissue of patients with Lyme arthritis (22). This suggests that other factors including, but not limited to localized tissue expression levels and the presence of potent APCs, as well as recruitment of T cells into inflamed tissue *via* chemokines, all of which are known to occur in the joint of subjects with Lyme arthritis, may drive neo-antigenicity of thymidine phosphorylase peptides ultimately leading to the activation of self-reactive T cells.

Apolipoprotein B-100 (apoB-100) is a major protein source of autoantigenic peptides in rheumatoid arthritis, atherosclerosis, systemic lupus erythematosus (SLE), and Lyme arthritis (23). Specifically, peptides p45 and p210 corresponding to residues 661–680 and 3,136–3,155, respectively in the protein, have been implicated in the immunogenicity of apoB-100 (38). Healthy donors in our cohort presented nested sets of the autoantigenic Lyme arthritis peptide ^{655}IEGNLIFDPNNYLPK^{669}, which overlaps with the p45 peptide from residue 661–669 (FDPNNYLPK). Accordingly, we speculate that this sequence overlap identifies the minimal autoreactive peptide. Speculation surrounding potential mechanisms responsible for local loss of tolerance to apoB-100 suggest that Th1 responses at sites of inflammation supply the necessary signals for increased expression of co-stimulatory molecules, leading to increased antigen presentation and ultimate loss of tolerance (39). Given the central role dendritic cells play in driving T cell responses in secondary lymphoid compartments, it is tempting to speculate that specific self-peptides become immunogenic, under infection-induced or inflammatory conditions, driving dysregulated adaptive immune responses that breach the threshold of established tolerance mechanisms.

One of the most commonly presented proteins in our assay was vimentin, a member of the intermediate filament family of the cellular cytoskeleton, which plays a crucial role in the pathogenesis of various inflammatory and autoimmune diseases including: rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondyloarthritis, among others (40). Antigenic hallmarks of vimentin, among other recognized autoantigens in autoimmune diseases, are exacerbated by post translational modifications, specifically citrullination. The role of protein citrullination in the context of Lyme arthritis remains relatively unexplored and deserves future examination, given that the *HLA-DRB1* alleles 01:01 and 04:01, risk alleles in Lyme arthritis, contain the shared epitope, a five-residue sequence in the HLA-DRβ chain associated with severe rheumatoid arthritis (41). In addition, the function and abundance of vimentin and other autoantigens have been implicated in their potential to become immunogenic in inflammatory settings, warranting further exploration.

Overall, our study contributes novel insights to understanding the interaction between dendritic cells and the *B. burgdorferi* spirochete. Our results corroborated known aspects of class II MHC presentation, profiled the HLA-DR self-immunopeptidome presented during *Borrelia* challenge, and identified sets of unique self-peptides derived from proteins associated with distinct biological processes and cellular compartments following exposure of mo-DCs to the live spirochete. Importantly, we advanced our understanding of the biological processes occurring in dendritic cells from healthy donors during *Borrelia* infection, which may shed light into mechanisms that promote the range of disease outcomes, including Lyme arthritis and PTLDS.

DATA AVAILABILITY STATEMENT

The list of all self-peptides identified under all conditions is provided as Supplemental Material (**Supplementary List 3**). Peaks files and .raw files are stored on a secure Johns Hopkins University One Drive server and will be made available to interested investigators. Please contact the corresponding author to obtain access.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MG-H and MS contributed to the conception and design of the study. ET and ED contributed to the conception of the natural antigen processing assay. RC and RO'M contributed to optimization and collection of LC-MS/MS data. All authors contributed to manuscript preparation as well as read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.00568/full#supplementary-material

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Role of HK2 in the Enzootic Cycle of *Borrelia burgdorferi*

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The two-component response regulator Rrp2 is a key activator controlling the production of numerous virulence factors of *Borrelia burgdorferi*, the Lyme disease pathogen. Previously it was shown that the cognate histidine kinase HK2 is not required for Rrp2 activation *in vitro*, nor for mammalian infection upon needle inoculation, raising the question whether HK2 has any role in the enzootic cycle of *B. burgdorferi*. In this study, we demonstrated that HK2 is not required for spirochetal survival in the tick vector. When fed on naive mice, the *hk2* mutant had reduced infectivity through the route of tick bite, suggesting that the spirochetes lacking HK2 had a disadvantage in the enzootic cycle. Furthermore, overexpression of *hk2* reduced the level of Rrp2 phosphorylation, suggesting that HK2 can function as a phosphatase to dephosphorylate Rrp2. Strains overexpressing *hk2* impaired the expression of RpoN regulon whose activation is dependent on Rrp2 phosphorylation and activation, and had reduced infectivity in mice. Taken together, these results demonstrate that although HK2 does not play an essential role in Rrp2 activation, it is important for the optimal fitness of *B. burgdorferi* in the enzootic cycle.

Keywords: lyme disease, *Borrelia* (Borreliella) *burgdorferi*, two-component system (TCS), Rrp2-HK2, OspC

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INTRODUCTION

The Lyme disease pathogen, *Borrelia burgdorferi, B. afzelii,* and *B. garinii*, is maintained in nature in two drastic different hosts, an *Ixodes* tick and a mammalian host. As an obligate parasite with a small genome, *B. burgdorferi* has evolved using its limited signaling and regulatory repertoire to adapt to both host environments. Comparing to free living bacteria such as *Escherichia coli,* which has more than 30 two-component signal transduction systems (TCSs), *B. burgdorferi* has reduced to two sets of TCS, HK1/Rrp1 and HK2/Rrp2 (in addition to the chemotactic CheA-CheY system) and has evolved to employ these two TCSs to survive in each of the two hosts encountered in the enzootic cycle. HK1/Rrp1, a c-di-GMP producing system, controls spirochete's adaptation to the tick vector (1–7), whereas HK2/Rrp2 is essential for *B. burgdorferi* to establish infection in the mammalian host (8–11).

The function of HK2/Rrp2 is largely based on the study of the response regulator Rrp2. A typical TCS consists of a histidine kinase as a sensor and a corresponding response regulator that mediates the cellular response (12). Rrp2 is a member of NtrC family transcriptional activator. It contains three putative functional domains: an N-terminal response regulator receiver

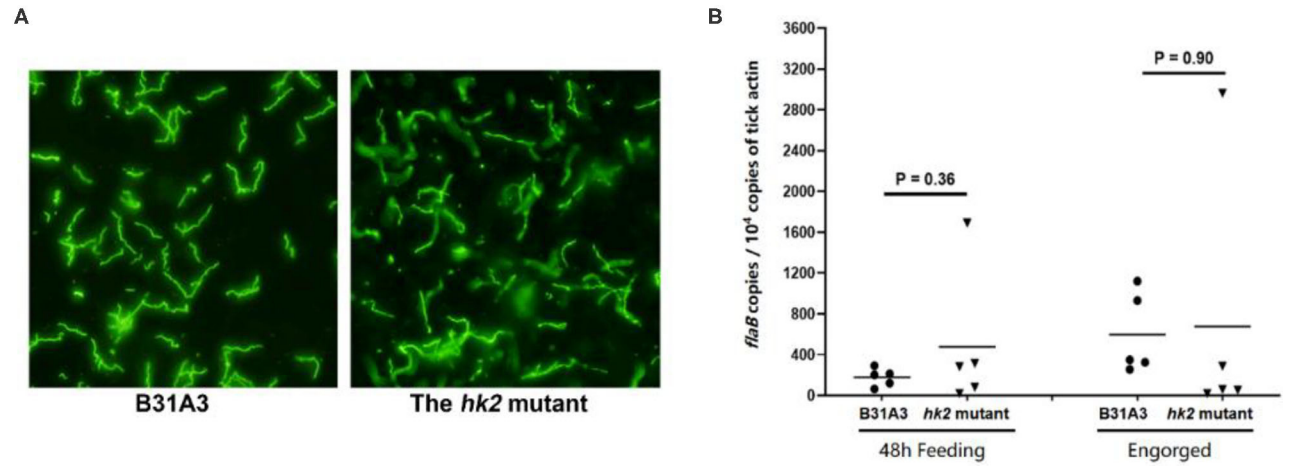

FIGURE 1 | IFA and qPCR analyses of spirochetes in fed larvae. **(A)** Unfed *I. scapularis* larvae harboring either the wild type B31A3 or the *hk2* mutant were fed on naive C3H/HeN mice, and engorged larvae were subjected to IFA analysis using fluorescein isothiocyanate-labeled anti-*B. burgdorferi* antibody. Five ticks were examined for each group, and a representative image for each group of ticks is shown. **(B)** 48 h feeding larvae or fully fed larvea were collected and subjected to qPCR analyses. Each data points were generated from the DNA sample extracted from three larvae. The copy numbers of the *flaB* gene of *B. burgdorferi* was used for caculating spirochetal numbers, which is nurmalized by 10^4 copies of the tick actin gene.

domain, a central activation domain, and a C-terminal helix-turn-helix (HTH) DNA-binding domain (38). The central activation domain of Rrp2 is highly conserved among a group of transcriptional activators (bacterial enhancer-binding proteins) that specifically activate genes from alternative sigma factor 54 (RpoN or σ^{54})-type promoter. The only gene with a σ^{54}-type promoter in *B. burgdorferi* identified to date is *rpoS,* which encodes the second alternative sigma factor RpoS (σ^S). Thus, upon activation by phosphorylation at its N-terminal receiver domain, Rrp2 activates its central domain, leading to activation of an alternative sigma factor cascade, σ^{54}-σ^S cascade (also called RpoN-RpoS pathway or Rrp2-RpoN-RpoS pathway) in *B. burgdorferi* [for review, see (13–15)]. Rrp2, along with a transcriptional activator BosR and a repressor BadR, regulates σ^{54}-σ^S sigma factor cascade, which in turn controls *ospC*, *dbpB/A*, *bbk32*, and many other mammalian infection-associated genes (8–11, 16–26).

Relative to the downstream targets controlled by Rrp2, the upstream event that activates Rrp2 is poorly understood. The gene *hk2* (BB0763) is adjacent to *rrp2* (BB0764) in the genome and purified HK2 is capable of phosphorylating Rrp2 *in vitro*, making HK2 qualified as the cognate histidine kinase for Rrp2 (8, 27). However, we and others previously showed that disruption of *hk2* does not affect *ospC* expression and activation of σ^{54}-σ^S cascade, suggesting that HK2 is not essential for Rrp2 phosphorylation (27, 28). The *hk2* mutant is also capable of infecting mice upon needle infection. This raises question whether HK2 plays any role in the enzootic cycle of *B. burgdorferi*. In this study, we further investigated the *hk2* mutant phenotype in the tick phase of the enzootic cycle, showing that the *hk2* mutant had a reduced infection via tick infestation. We also took another approach to study HK2 functions by overexpressing *hk2*. The results showed that HK2 is functional and important for maximum fitness in the enzootic cycle of *B. burgdorferi*.

RESULTS

HK2 Is Not Required for Spirochete Survival During Tick Feeding

Previously we showed that the *hk2* mutant had normal infectivity in mice upon needle inoculation (28). To examine whether HK2 plays a role in tick phase of the enzootic cycle, naive *Ixodes scapularis* larvae were fed on immunocompetent C3H/HeN mice that were needle-infected with wild-type or the *hk2* mutant spirochetes. During and after depletion, engorged larvae were then subjected to immunofluorescent assay (IFA) (**Figure 1A**) and qPCR analyses (**Figure 1B**) to assess the spirochetal numbers. The result showed that there was no significant difference in spirochetal numbers in the tick midguts at 48 h during feeding or after feeding on mice infected with wild-type and the *hk2* mutant *B. burgdorferi*, suggesting that HK2 is not required for spirochetal survival during tick feeding (**Figures 1A,B**).

The *hk2* Mutant Has Normal Level of Activation of σ^{54}-σ^S Sigma Factor Cascade During Tick Feeding

We and others showed that HK2 is not required for Rrp2 activation and σ^{54}-σ^S cascade activation under the *in vitro* cultivation conditions (27, 28). In the enzootic cycle of *B. burgdorferi*, spirochetes colonizing in the midgut of unfed ticks begin to replicate and activate σ^{54}-σ^S cascade when ticks feed (14). Whether HK2 is required for σ^{54}-σ^S cascade activation during tick feeding has not been examined. Thus, infected fed

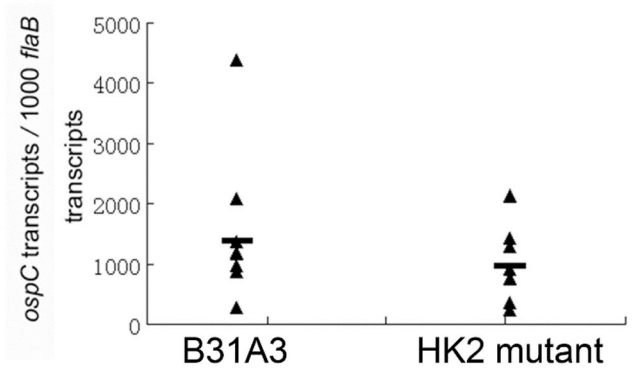

FIGURE 2 | qRT-PCR analyses of spirochetes during tick feeding. Flat *I. scapularis* nymphs infected with either the wild-type strain B31A3 or the *hk2* mutant were fed on naive C3H/HeN mice, and engorged nymphs were subjected to qPCR analyses. Seven ticks were examined for each group, and each data point was from one nymph. The level of *ospC* expression were nornalized with the level of *flaB* transcripts of *B. burgdorferi*. No significant difference was observed between the two groups.

TABLE 1 | The infectivity of the *hk2* mutant via tick bite¶.

Strains	No. of mouse tissues culture positive/total No. of tissues tested			No. of tissues infected/total No. of tissues
	Skin	Joint	Heart	
WT (B31A3)	13/15	13/15	13/15	39/45 (86.7%)*
The *hk2* mutant	8/20	11/20	10/20	29/60 (48.3%)*

¶*Dose of infection: 5 nymphs per mouse.*

**The p-value between the two group is 0.04 (Fisher's Exact Test).*

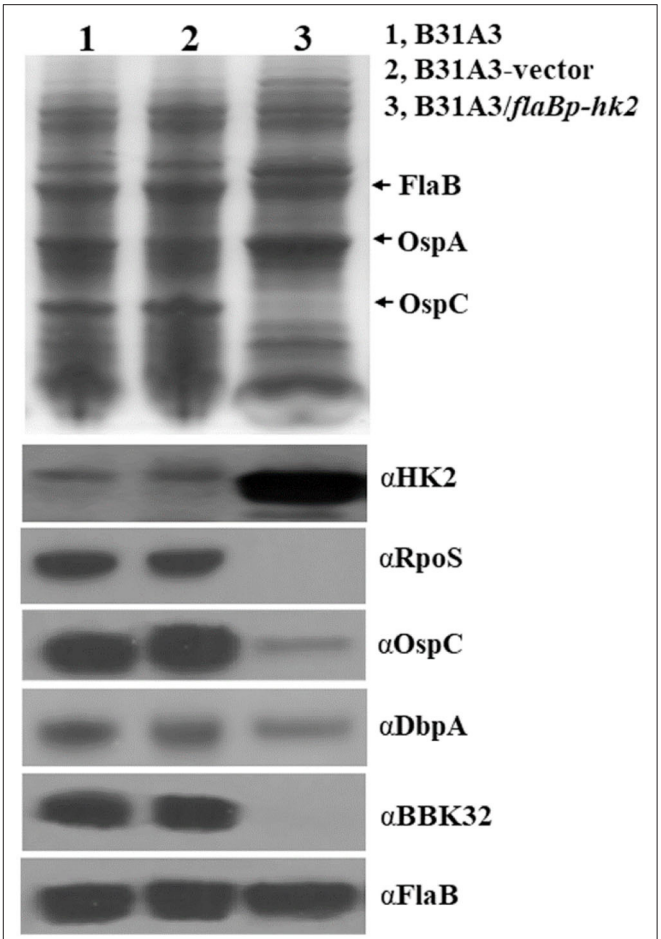

FIGURE 3 | Overexpression of HK2 impaired production of RpoS and RpoS-controlled lipoproteins. Spirochetes were cultivated in BSK-II medium at 37°C and were harvested at the stationary phase, and whole-cell lysates were subjected to SDS-PAGE (top) and Western blot analyses (bottom). The positions of proteins and antibodies used are indicated on the right. B31A3, wild-type *B. burgdorferi*; B31A3-vector, B31A3 carrying an empty shuttle vector; B31A3/*flaBp*-HK2, B31A3 carrying a vector harboring a *hk2* gene driven by a constitutive *flaB* promoter.

larvae from above experiments were allowed to molt to nymphs. Flat nymphs were fed on naive mice. Engorged nymphs were collected for quantitative RT-PCR analysis to determine the levels of ospC expression, a surrogate for σ^{54}-σ^{S} cascade activation. As shown in **Figure 2**, there was no significant difference in levels of ospC expression between wild-type spirochetes and the *hk2* mutant during tick feeding, suggesting that HK2 is not required for σ^{54}-σ^{S} cascade activation *in vitro* as well as during tick feeding.

The *hk2* Mutant Has Reduced Infectivity via Tick Infestation

Although HK2 is not required for mammalian infection via needle inoculation using *in vitro* cultured spirochetes, it remains to be determined whether HK2 plays a role via tick infestation. Accordingly, flat nymphs harboring wild-type or the *hk2* mutant spirochetes were allowed to feed on groups of naive C3H/HeN mice. Three weeks after tick bites, mice were sacrificed, and tissue biopsies including skin, joint, and heart were collected for culturing the presence of spirochetes. While 86.7% of mouse tissues infected with ticks harboring wild-type *B. burgdorferi* were culture positive, 48.3% mouse tissues infected with ticks harboring the *hk2* mutant were culture positive (**Table 1**), suggesting that the *hk2* mutant has reduced infectivity via the route of tick infection.

Overexpression of *hk2* Impairs Activation of σ^{54}-σ^{S} Sigma Factor Cascade

To further investigate the function of HK2, we took another approach by overexpressing *hk2* by transforming *B. burgdorferi* with a shuttle vector carrying a *hk2* gene driven by a constitutive *flaB* promoter. The shuttle vectors were transformed into wild-type strain B31A3. As expected, transformed clones carrying a *flaB* promoter-driven *hk2* had a much higher level of Hk2 than that of wild-type strain (**Figure 3**). Overexpression of *hk2* dramatically reduced production of RpoS and RpoS-dependent

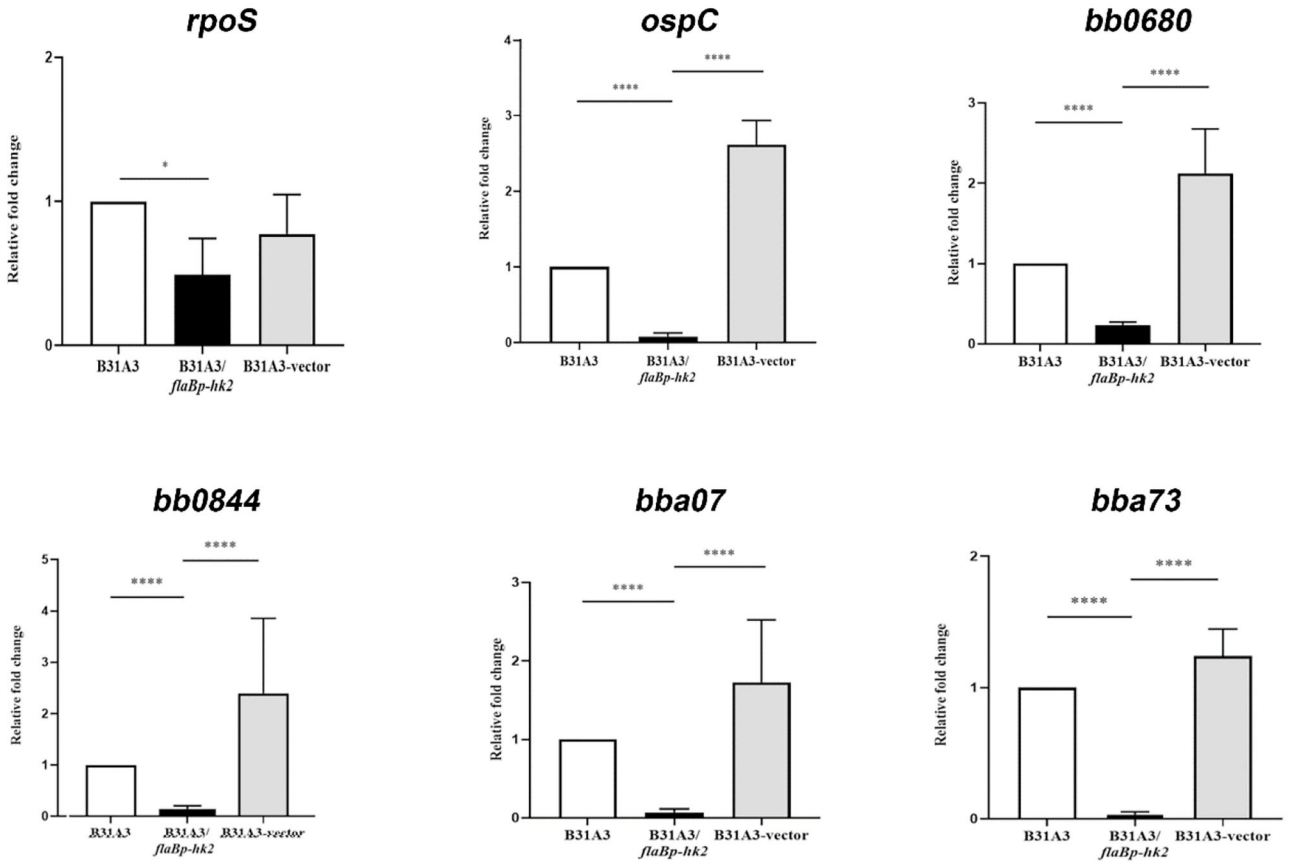

FIGURE 4 | qRT-PCR analyses of several RpoS-controlled genes. Spirochetes were cultured at 37°C in BSK-II medium and harvested in the late-log phase. RNAs were extracted and subjected to qRT-PCR analyses. Samples were first normalized with the *flaB* level, and then levels of gene expression were reported relative to that of wild-type B31A3 (with the level of expression of each gene in B31A3 as 1.0). *$p < 0.01$; ****$p < 0.0001$.

TABLE 2 | The infectivity of the *hk2* overexpression strain.

***Borrelia* strains and infection dose**	**No. of mouse tissues culture positive/total No. of tissues tested**			**No. of tissues infected/total No. of tissues**
	Skin	**Joint**	**Heart**	
1×10^5/mouse				
B31A3	3/3	3/3	3/3	9/9 (100%)
B31A3/*flaBp-hk2*	2/3	0/3	1/3	3/9 (33%)*
1×10^3/mouse				
B31A3	7/7	7/7	7/7	21/21 (100%)
B31A3/*flaBp-hk2*	0/7	4/7	0/7	4/21 (19%)§

**The p-value between the two groups is 0.16.*
§*The p value between the two groups is 0.01.*

surface lipoproteins such as OspC, DbpA, and BBK32 (**Figure 3**). Further qRT-PCR analyses showed that transcripts of *rpoS* and several RpoS-dependent genes (*ospC*, *bb0680*, *bb0844*, *bba07*, *bba73*) were significantly reduced upon HK2 overexpression (**Figure 4**). These results indicate that overexpression of *hk2* impaired the activation of σ^{54}-σ^{S} cascade.

Overexpression of *hk2* Reduces Infectivity

Given the importance of σ^{54}-σ^{S} cascade in mammalian infection, we examined the ability of the *hk2* overexpression strain to infect mice. Groups of immunocompetent C3H/HeN mice were inoculated with either a high dose (1×10^5 spirochetes per mouse) or a low dose (1×10^3 spirochetes per mouse) of wild-type *B. burgdorferi* B31A3 or the *hk2* overexpression strain B31A3/*flaBp-hk2*. Four weeks post-inoculation, mice were sacrificed, and various mouse tissues (skin, heart, and joint) were collected and cultured for spirochete growth. All mouse tissues from mice inoculated with wild-type *B. burgdorferi* B31A3 were culture positive, whereas mouse tissues from mice inoculated with B31A3/*flaBp-hk2* were 33% ($p = 0.16$) and 19% ($p = 0.01$) culture positive for high dose group and low dose group, respectively (**Table 2**), indicating that overexpressing *hk2* reduced the infectivity of *B. burgdorferi* in mice.

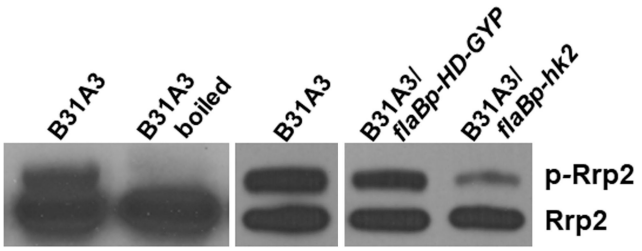

FIGURE 5 | Overexpressing HK2 reduces the level of phosphorylated Rrp2 in *B. burgdorferi*. Phos-tag SDS-PAGE and immunoblotting was used to detect both phosphorylated and dephosphorylated Rrp2 in the cell. Wild-type *B. burgdorferi* B31A3, B31A3 carrying a shuttle vector harboring a unrelated protein HD-GYP (B31A3/*flaBp-HD-GYP*), or B31A3 carrying a shuttle vector harboring a *hk2* gene driven by a *flaB* promoter GYP (B31A3/*flaBp-hk2*), were harvested at mid-log phase and cell lysates were prepared and separated on 7.5% SDS-PAGE containing 0, 5, 10, and 25 uM Phos-tag followed by immunoblotting using anti-Rrp2 antibody. p-Rrp2, the band corresponds to phosphorylated Rrp2. As a unphosphorylated Rrp2 control, B31A3 was also treated by boiling (lane 2) prior to Phos-tag SDS-PAGE (Rrp2 phosphorylation is unstable and sensitive to heat).

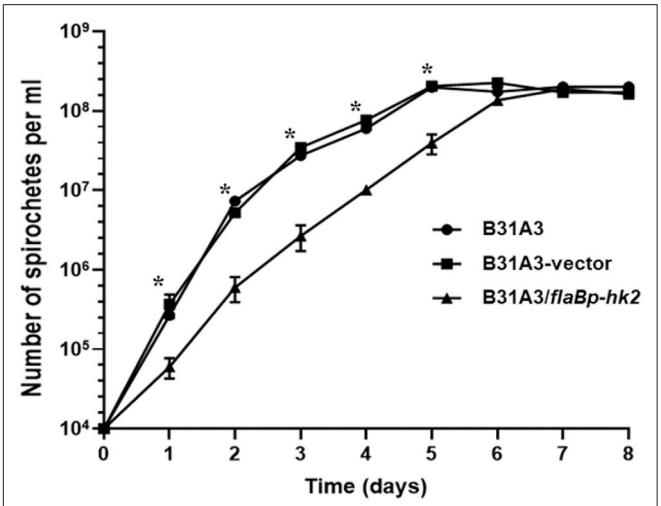

FIGURE 6 | HK2 overexpression resuls in reduced growth rate. Wild-type *B. burgdorferi* strain B31A3, B31A3 carrying an empty shuttle vector (B31A3-vector), or B31A3 carrying a shuttle vector harboring a *hk2* gene driven by a *flaB* promoter (B31A3/*flaBp-hk2*) were cultivated in standard BSK-II medium at 37°C with a initial cell density of 1×10^4 spirochetes/ml. Numbers of spirochetes were enumerated under a dark-field microscope. Each data point is the average of data from three independent cultures. *$p < 0.01$ (paired student test).

HK2 Functions as a Phosphatase of Rrp2

We further investigated possible mechanisms underlying the phenotypes of HK2 overexpression. Some two-component histidine kinases can function as phosphatase (29, 30). Given that activation of σ^{54}-σ^S cascade requires Rrp2 phosphorylation, we postulate that Hk2 may function as a phosphatase for Rrp2. Because aspartate phosphorylation of response regulators has short half-life and is very unstable, and antibodies that recognize phospho-Asp are not available, we performed Phos-tag™ acrylamide gel electrophoresis that uses dinuclear metal complex as a specific phosphate-binding agent to chelate phosphate (31, 32), a method that has shown to successfully separate phosphorylated and unphosphorylated forms of other response regulator proteins (33). Accordingly, *B. burgdorferi* lysates were subjected to Phos-tag SDS-PAGE followed by immunoblotting using anti-Rrp2 monoclonal antibody. Two distinct bands were observed in B31A3 (**Figure 5**, lane 1): the lower band corresponded to unphosphorylated Rrp2 and the top band corresponded to phosphorylated Rrp2 (p-Rrp2). When the cell lysate was treated by heat (boiling) prior to Phos-tag SDS-PAGE, the top band disappeared (**Figure 5**, lane 2), consistent with the fact that Asp-phosphorylation is unstable and heat sensitive to heat. The strain with HK2 overexpression dramatically reduced the intensity of the top band corresponding to phosphorylated Rrp2 (**Figure 5**, last lane), whereas the strain carrying the same shuttle vector that overexpressed an unrelated protein HD-GYP did not affect the level of Rrp2 phosphorylation (**Figure 5**, lane 4). This result suggests that although HK2 is not required for Rrp2 phosphorylation, it can function as a phosphatase to dephosphorylate Rrp2, and the impaired activation of σ^{54}-σ^S cascade by HK2 overexpression is, at least in part, due to the reduced level of Rrp2 phosphorylation.

We and others showed that Rrp2 phosphorylation is required for cell growth, in addition to activation of σ^{54}-σ^S cascade (34, 35). If HK2 overexpression reduces the level of Rrp2 phosphorylation, one would expect that it will affect spirochetal growth. Indeed, B31A3/*flaBp-hk2* displayed a distinct show growth rate than B31A3 and B31A3 carrying an empty shuttle vector (**Figure 6**). This observation further supports the hypothesis that HK2 functions as a phosphatase of Rrp2.

DISCUSSION

The Rrp2-RpoN-RpoS pathway, or the σ^{54}-σ^S alternative sigma factor cascade, is the most studied regulatory pathway in *B. burgdorferi* (13, 14). It plays a major role in controlling differential gene expression during the process of the spirochetal transmission from ticks to mammals and has thus been called "Gatekeeper" (11). Therefore, understanding how this pathway is activated is important for our understanding of how *B. burgdorferi* migrates between ticks and mammals. It has been perplexing that HK2, being the cognate histidine kinase of Rrp2, showed no effect on activation of σ^{54}-σ^S cascade *in vitro* and is dispensable for mammalian infection via the route of needle inoculation (27, 28). Given that *B. burgdorferi* has a compact genome and that the *hk2* gene is highly conserved among all *B. burgdorferi* strains including *B. garinii* and *B. afzelii*, it is unlikely that *hk2* is no longer needed for *B. burgdorferi* and is in the process of gene loss through genome reduction. In this study, we showed that strain lacking HK2 reduced infectivity via tick bites, the nature route of infection. Tightly controlled *hk2* expression is also important for mammalian infection, as HK2 overexpression led to reduced infectivity in mice. We also

successfully employed the Phos-tag method, which not only allowed us to detect phosphorylated form Rrp2 but also showed that although HK2 is not required for Rrp2 phosphorylation *in vitro*, it can function as a phosphatase that dephosphorylates Rrp2. Together, this study demonstrates that HK2 is not what was previously perceived dispensable for the pathogenesis of *B. burgdorferi*; rather, it plays an important role in the enzootic cycle of *B. burgdorferi*.

The observation that the *hk2* mutant showed different infection outcomes between needle inoculation vs. tick bite underlines the importance of using nature route of tick infestation for assessment of infectivity of a *B. burgdorferi* mutant. It has been reported that different route of infection by *B. burgdorferi* can have different infectivity and tissue tropism (36, 37). For example, the *dbpBA* mutant lacking decorin-binding proteins A and B showed avirulent phenotype by needle-inoculation, but later was demonstrated to be fully infectious via tick infestation (36). One of the obvious reasons for such different outcomes is that spirochetes cultivated *in vitro* used for needle inoculation have different gene expression profile from that in ticks. In this regard, we examined the *ospC* expression of the *hk2* mutant during tick feeding, as *ospC* expression is the surrogate for activation of σ^{54}-σ^{S} cascade (**Figure 2**). This is important because the previous conclusion that HK2 is not required for Rrp2-RpoS-RpoS activation is based on spirochetes grown *in vitro*. Whether HK2 plays a role in Rrp2-RpoS-RpoS activation *in vivo* has not been examined. Based on the result from the current study, we now can conclude that HK2 is not essential for Rrp2-RpoS-RpoS activation *in vivo*, i.e., during tick feeding. This data also indicates that the reduced infectivity of the *hk2* mutant via tick bite was not due to a defect in activation of σ^{54}-σ^{S} cascade, suggesting HK2 may influence other pathways or genes. For instance, HK2 may regulate expression of genes important for spirochetal migration from the tick midgut to salivary gland, whereas needle inoculation bypasses such requirement.

Our results show that Hk2 can function as a phosphatase to dephosphorylate Rrp2, which could explain why Hk2 overexpression resulted in an impaired activation of σ^{54}-σ^{S} cascade and reduced infectivity in mice. However, this observation does not exclude other effects of HK2 overexpression that might also contribute to the phenotype. For example, HK2 overexpression might sequester potential HK2 binding ligand or interfere the interacting partner of Rrp2 such as RpoN and possibly BosR. One caveat of this study is that the phenotype of HK2 overexpression in ticks was not examined. B31A3/*flab-hk2* is defective in mice, which hampered us feeding ticks on infected mice. Further study using artificial feeding to infect ticks is warranted to confirm the HK2 overexpression phenotype in ticks. Nevertheless, although a lot needs to be learned about HK2 function, this work demonstrates, for the first time, that the *hk2* mutant is defective in the enzootic cycle of *B. burgdorferi*, and HK2 can function as a phosphatase for Rrp2. That is, despite the fact that HK2 is not required for Rrp2 phosphorylation *in vitro*, HK2 is important to the enzootic cycle of *B. burgdorferi* and further studies are warranted to elucidate the function of HK2 including the role of the putative PAS domain, the signal HK2 may senses, and the nature of the defect of the *hk2* mutant in the enzootic cycle.

MATERIALS AND METHODS

B. burgdorferi Strains and Culture Conditions

Low-passage, virulent *B. burgdorferi* strain B31A3 (a gift from Dr. Patricia Rosa, Rocky Mountain Laboratories, NIH) was used in this study. A B31A3 derived *hk2* mutant used in this study was constructed previously by our laboratory (28). Spirochetes were cultivated in Barbour-Stoenner-Kelly (BSK-II) medium supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, AR) (38) at 37°C with 5% CO_2. For the HK2 overexpression *B. burgdorferi* strain, 300 μg/ml of kanamycin antibiotics was added to the cultures. The constructed shuttle vector (pHX55-HK2) was maintained in *Escherichia coli* strain DH5α.

Construction of the Strain With Overexpression of *hk2*

For *hk2* overexpression, the PCR fragments of the wild-type *hk2* gene and the *flaB* promoter were fused at the ATG site, and the combined fragment was then cloned into the *BamH*1 and *Pst*I sites of the shuttle vector pJD55 (36), resulting in pHX55-HK2. The constructed shuttle vector was then transformed into B31A3, and kanamycin-resistant *Borrelia* transformants were confirmed by PCR for the presence of pHX55-HK2 and by Western blot for HK2 overproduction. Plasmid profiles of the confirmed transformants were determined by multiple PCR analyses for each of the endogenous plasmids as described previously (39, 40). One of the HK2 overexpression clones that had plasmid profiles identical to parental B31A3 was chosen for further study.

Mouse Infection With *B. burgdorferi* via Needle Inoculation

All mouse experiments were approved by the IACUC committee of Indiana University School of Medicine (IUSM) under the protocol number #11339. Four-week-old C3H/HeN mice (Harlan, Indianapolis, IN) were subcutaneously inoculated with doses of spirochetes as indicated. Mice were euthanized at the end of the experiments, and multiple tissues (joint, heart, skin) were harvested. All tissues were cultivated in 2 ml of the BSK-II medium (Sigma-Aldrich, St. Louis, MO) containing an antibiotic mixture of phosphomycin (2 mg/ml), rifampin (5 mg/ml), and amphotericin B (250 mg/ml) (Sigma-Aldrich) to inhibit bacterial and fungal contamination. All cultures were maintained at 37°C and examined for the presence of spirochetes by dark-field microscopy beginning from 5 days after inoculation. A single growth-positive culture was used as the criterion to determine positive mouse infection.

Tick-Mouse Cycle of *B. burgdorferi*

Ixodes scapularis egg masses were purchased from Oklahoma State University. The tick-mouse experiments were conducted in IUSM and approved were approved by the IACUC committee of

IUSM under protocol number #11339. Unfed larvae were fed on groups of mice (C3H/HeN, three mice per group, 150–200 larvae per mouse) that were needle infected with spirochetes. Ticks were allowed to feed to repletion (3–4 days) and then collected within 24 h. A portion of fed larvae were subjected to analyses. The remaining fed larvae were maintained in the tick incubator and allowed to molt to the nymphal stage (about 5 weeks). One month after molting, unfed nymphs were then allowed to feed on naive C3H/HeN mice. Fully engorged nymphal ticks were collected within 24 h of repletion and subjected to analyses. Mice infected with tick bites were subjected to infection analyses as described above.

Immunofluorescence Assay (IFA)

IFA was performed as reported previously (3). Briefly, the entire contents of a fed tick were smeared and fixed on silylated microscope slides (CEL Associates, Pearland, TX). The slides were incubated with BacTrace fluorescein isothiocyanate-conjugated goat anti-*B. burgdorferi* antibody (Kirkegaard and Perry Laboratories Gaithersburg, MD) at 37°C. Samples were observed using an Olympus BX50 fluorescence microscope. Twenty ticks from each group were examined by IFA.

qPCR and qRT-PCR

For qPCR analyses of *B. burgdorferi* DNA in ticks, DNA samples were extracted from ticks using a DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. qPCR was performed with primer pairs of qflaB-F/R and qTactin-F/R as described previously (41). Calculations of relative DNA copy number (represented by *flaB*) were normalized with the copy number of the tick actin gene.

For quantification of *ospC* transcripts of *B. burgdorferi* in ticks, RNA samples were extracted from ticks using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. To reduce trace amounts of DNA contamination, samples were further digested with RNase-free DNaseI (Qiagen), purified using the RNeasy mini kit (Qiagen), and analyzed with NanoDrop One^C Spectrophotometer (Thermo Fisher Scientific). DNA-free RNA was confirmed by PCR amplification for the *B. burgdorferi flaB* gene. cDNA was synthesized using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa). Given the low levels of bacterial RNA in ticks, the specific primers for each gene target were used for cDNA synthesis instead of random primers previously (41). To quantify the transcript levels of genes of interest, an absolute quantitation method was used to create a standard curve for the qPCR assay according to the manufacturer's protocol (Strategene, La Jolla, CA). Briefly, the PCR product of the *flaB* gene served as a standard template. A series of 10-fold dilutions (10^2-10^7 copies/ml) of the standard template was prepared, and qPCR was performed to generate a standard curve by plotting the initial template quantity against the Ct values for the standards. The quantity of the targeted genes in the cDNA samples was calculated using their Ct values and the standard curve. The samples were assayed in triplicate using the ABI 7000 Sequence Detection System and PowerUp SYBR Green Master Mix (Applied Biosystems). The levels of the target gene transcript were reported as per 1000 copies of *flaB*.

For qRT-PCR analyses of gene expression in cultured *B. burgdorferi*, spirochetes were cultured at 37°C in BSK-II medium and harvested in the late-log phase. RNAs were extracted and subjected to qRT-PCR analyses as described above. Primers used for *rpoS*, *ospC*, *bb0680*, *bb0844*, *bba07*, *bba73* were described previously (42). Samples were first normalized with the *flaB* level, and then levels of gene expression were reported relative to that of wild-type B31A3 (with the level of expression of each gene in B31A3 as 1.0).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

Spirochetes from mid-log cultures were harvested by centrifugation at 8,000 × g for 10 min and washed three times with PBS (pH 7.4) at 4°C. Pellets were suspended in SDS buffer containing 50 mM Tris-HCl (pH 8.0), 0.3% sodium dodecyl sulfate (SDS), and 10 mM dithiothreitol (DTT). Cell lysates (10^8 cells per lane) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (GE-Healthcare, Milwaukee, WI). Membranes were blotted with either single or a mixed monoclonal/polyclonal antibodies against HK2 (28), RpoS, OspC, DbpA, BBK32 (43), followed with goat anti-mouse or anti-rat lgG-HRP secondary antibody (1:1,000; Santa Cruz Biotechnology). Detection of horseradish peroxidase activity was determined by the enhanced chemiluminescence method (Thermo Pierce ECL Western Blotting Substrate) with subsequent exposure to X-ray film.

Phos-Tag SDS-PAGE

Spirochetes were grown at mid-log phase and harvested by centrifugation for 1 min at 4°C. Cell pellets were washed twice with ice-cold PBS and resuspended in 1 x SDS-PAGE sample buffer. To get rid of the cell debris, the samples were centrifuged at 4°C for 3 min. The supernatants were then loaded on 7.5% Phos-tag SDS-PAGE gels with or without Phos-tag (33). Phos-tag acrylamide AAL-107 was purchased from Wako Chemicals USA. To prepare the control sample in which all Rrp2 molecules are dephosphorylated, cell lysates were boiled for 5 min prior to loading into Phos-tag gel. The gels were run in MOPS buffer (100 mM Tris-HCl, 100 mM MOPS, 0.1% SDS, and 5 mM sodium bisulfite) at 100V, 4°C followed by treatment with transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol) containing 1 mM EDTA at room temperature with gentle shaking for 15 min to remove the zinc from the gel. The gel was further washed in new transfer buffer without EDTA for 15 min at room temperature with gentle shaking. Separated proteins in the gels were transferred onto NC or PVDF membranes for immunoblot.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the IACUC committee of Indiana University School of Medicine (IUSM) under the protocol number # 11339.

AUTHOR CONTRIBUTIONS

QL and HX performed the experiment and wrote the paper. YZha and JY performed the experiment. JD performed the experiment and data analysis. YZho analyzed the data and edits the paper. XY and YL designed and wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CORRECTION
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Corrigendum: Role of HK2 in the Enzootic Cycle of *Borrelia burgdorferi*

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A Corrigendum on

Role of HK2 in the Enzootic Cycle of *Borrelia burgdorferi*
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In the original article, there was a mistake in **Figure 5** as published. Due to an error in compiling multi-panel images, a gap between the image of "B31-A3" and the image of "B31A3/flaBp-HD-GYP; B31A3/flaBp-hk2" was omitted. In this figure, unphosphorylated Rrp2 (lower lane) serves as an internal control for each sample. Overproduction of an unrelated protein HD-GYP (B31A3/flaBp-HD-GYP) serves as the negative control for overproduction of Hk2 (B31A3/flaBp-hk2), showing a reduction of Rrp2 phosphorylation by overexpression of Hk2. The corrected **Figure 5** appears below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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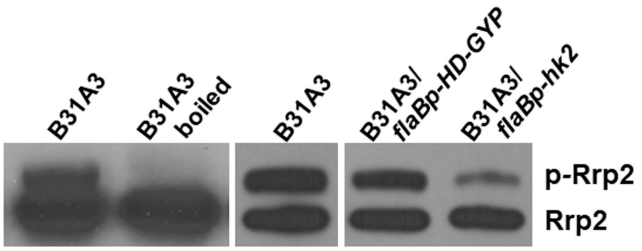

FIGURE 5 | Overexpressing HK2 reduces the level of phosphorylated Rrp2 in *B. burgdorferi*. Phos-tag SDS-PAGE and immunoblotting was used to detect both phosphorylated and dephosphorylated Rrp2 in the cell. Wild-type *B. burgdorferi* B31A3, B31A3 carrying a shuttle vector harboring a unrelated protein HD-GYP (B31A3*/flaBp-HD-GYP*), or B31A3 carrying a shuttle vector harboring a *hk2* gene driven by a *flaB* promoter GYP (B31A3*/flaBp-hk2*), were harvested at mid-log phase and cell lysates were prepared and separated on 7.5% SDS-PAGE containing 0, 5, 10, and 25 uM Phos-tag followed by immunoblotting using anti-Rrp2 antibody. p-Rrp2, the band corresponds to phosphorylated Rrp2. As a unphosphorylated Rrp2 control, B31A3 was also treated by boiling (lane 2) prior to Phos-tag SDS-PAGE (Rrp2 phosphorylation is unstable and sensitive to heat).

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BRIEF RESEARCH REPORT
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Borrelia miyamotoi Serology in a Clinical Population With Persistent Symptoms and Suspected Tick-Borne Illness

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Eighty-two patients seeking consultation for long-term sequalae after suspected tick-borne illness were consecutively tested for *Borrelia miyamotoi* antibodies using a recombinant glycerophosphodiester phosphodiesterase (GlpQ) enzyme immunoassay. Twenty-one of the 82 patients (26%) tested positive on the GlpQ IgG ELISA. Nearly all of the patients (98%) had no prior *B. miyamotoi* testing, indicating that clinicians rarely test for this emerging tick-borne pathogen. Compared to patients who solely tested positive for Lyme disease antibodies, patients with *B. miyamotoi* antibodies presented with significantly more sleepiness and pain. A prospective study is needed to ascertain the relationship between the presence of *B. miyamotoi* antibodies and persistent symptoms.

Keywords: *Borrelia miyamotoi* disease, relapsing fever borrelia, Lyme disease, serodiagnosis, post-treatment Lyme disease syndrome, borreliosis

INTRODUCTION

Borrelia miyamotoi is a relapsing fever spirochetal bacterium first identified in Japan in 1994 (1). The first human cases were reported in Russia in 2011 (2), and in the Northeastern United States in 2013 (3). *B. miyamotoi* is transmitted by the same hard-bodied ticks (*Ixodes* species) that are vectors of *Borrelia burgdorferi* (3, 4).

B. miyamotoi infection is clinically similar to Lyme disease (5) with manifestations of fever, fatigue, headache, myalgia, chills, and nausea (2, 4, 6, 7). Like Lyme disease, *B. miyamotoi* infection can lead to significant neurologic complications (5). However, unlike Lyme disease, erythema migrans rash, and arthralgias are uncommon (2). Overall, patients with acute *B. miyamotoi* infection often present with more severe symptoms, especially headaches and fever, than patients with acute Lyme disease (2). Meningoencephalitis can also occur in immunocompromised patients (2, 4). Relapsing fevers occur

in a subset of patients with *B. miyamotoi* disease (2, 5). Like Lyme disease, *B. miyamotoi* disease is treated with 2–4 weeks of antimicrobial therapy—most often doxycycline or amoxicillin (2, 8, 9).

Polymerase chain reaction (PCR) analysis is used to confirm acute *B. miyamotoi* infection (9). Seroconversion is assessed using the glycerophosphodiester phosphodiesterase (GlpQ) enzyme immunoassay; IgM antibodies have been found to be reactive between 11 and 20 days after disease onset, and IgG antibodies reactive 21–50 days after disease onset (4). The GlpQ protein is present among all relapsing fever spirochetes, but absent in *B. burgdorferi* (4, 10). PCR positivity rates have ranged from 17% among patients hospitalized for acute infection with suspected tick-borne disease in Russia (2), to 0.7–0.8% (3, 6) among acutely ill patients in the Northeastern United States. A recent study in France showed that 43 of 824 patients (5.22%) with polymorphic signs and symptoms and suspected tick-borne illness were PCR positive for *B. miyamotoi* (5). Studies in North America using the GlpQ assay among patients with presumed tick-borne infection have revealed antibody seropositivity rates ranging from 3 to 21% Northeast (11), Canada (12), and California (13).

Although persistent neurocognitive and musculoskeletal complaints have been widely described among a subset of patients with Lyme disease (11), no research to date has investigated whether persistent or atypical symptoms can occur after *B. miyamotoi* infection (7). It is also unknown what proportion of patients in Lyme-endemic areas experience symptoms suggestive of tick-borne illness, but have negative diagnostic tests for Lyme disease and therefore do not receive antimicrobial treatment. This study is the first to investigate whether patients with chronic symptoms seeking consultation for suspected tick-borne illness show evidence of prior exposure to *B. miyamotoi*. In this report, we compare the prevalence and clinical characteristics of patients with positive serology to *B. miyamotoi* to those with *B. burgdorferi*.

METHODS

This study included 82 patients consecutively screened for *B. miyamotoi* and *B. burgdorferi* antibodies as part of a clinical workup for tick-borne illness at the Columbia University Irving Medical Center from June 2017 to October 2018. Chart review was approved by the New York State Psychiatric Institute IRB. Clinic patients were seeking a second opinion to determine whether their persistent polymorphic symptoms were attributable to tick-borne infection. Patients frequently endorsed chronic symptoms of fatigue, pain, neurocognitive, and psychiatric problems.

Evaluations consisted of comprehensive physician assessments, serologic testing, and questionnaires. The GLP-Q assay was an indirect EIA for detection of antibody to *B. miyamotoi*, performed at Imugen, Inc. in Norwood, MA. As previously reported, they use the GlpQ gene sequence from B. miyamotoi (GenBank accession number AY368276) as the basis for cloning and expression as a 38-kDa recombinant protein (rGlpQ) (6). *B. burgdorferi* antibodies were assessed using the C6 peptide ELISA and IgM and IgG western immunoblot. Patients were designated *B. miyamotoi* positive based on IgG or IgM GlpQ seroreactivity (defined as >1 to the value calculated for the highest result on the standard curve). Patients were designated Lyme-positive if they met 2017 CDC surveillance criteria for definite or probable Lyme disease, having an EM skin lesion or multisystem clinical symptoms with at least 5 positive IgG bands on the Western blot.

Patients rated their symptoms using the General Symptom Questionnaire-30 (GSQ-30), a measure specifically developed to assess multisystem symptom burden in patients with early Lyme disease and post-treatment Lyme disease syndrome (12). Patients also completed the Beck Depression Inventory-II, Cognitive Failures Questionnaire, Fatigue Severity Scale, Epworth Sleepiness Scale, McGill VAS Pain Scale, and Zung Anxiety Scale. Mann-Whitney *U* tests were conducted to compare *B. miyamotoi*-positive and *B. burgdorferi*-positive patients on these measures.

RESULTS

Of the 82 patients, 21 (26%) tested positive for *B. miyamotoi* by anti-GlpQ ELISA; all were IgG positive and IgM negative. Of these 21 patients, five also met CDC surveillance criteria for definite or probable Lyme disease. Of the 61 patients who tested negative for *B. miyamotoi*, 22 met criteria for definite or probable Lyme disease. The remaining 39 patients tested negative for both *B. miyamotoi* and *B. burgdorferi* antibodies.

The *B. miyamotoi*-positive group reported significantly more sleepiness on the Epworth Sleepiness Scale ($Md = 9$ vs. 4, $U = 42.00$, $z = -2.51$, $p = 0.01$) and significantly more pain on the VAS ($Md = 5.80$ vs. 2.70, $U = 58.00$, $z = -2.02$, $p = 0.04$) than the group with probable or definite Lyme disease. On the total score and individual item level, there were no significant between-groups differences on the GSQ-30, but the *B. miyamotoi*-positive group did endorse being bothered more by headaches than the *B. burgdorferi*-positive group ($Md = 3$ vs. 2 at a trend level, $U = 52.50$, $z = -1.79$, $p = 0.07$).

Data for *B. miyamotoi*-positive patients is presented in **Supplementary Table 1**. Mean age was 34 years. Eight of 21 (38%) reported hospitalization (seven medical and one psychiatric) since symptom onset, three for cardiac and two for neurologic abnormalities. All 21 received prior antibiotic treatment, of whom 20 received at least 2 weeks of doxycycline or amoxicillin. Sixteen of the 21 patients lived in the Northeast/Mid-Atlantic USA. Of the remaining five patients, two lived in California, two lived in Florida, and one lived in Illinois. Of the 82 patients in the study, 80 (98%) had not been previously tested for *B. miyamotoi* infection. Among the 21 patients positive on the GlpQ ELISA, 18 were also tested using the Lyme C6 ELISA and none were positive.

DISCUSSION

This is the first study to investigate the presence of *B. miyamotoi* antibodies in a clinical population experiencing persistent

symptoms and suspected tick-borne illness. We found a high rate of *B. miyamotoi* GlpQ IgG antibody seropositivity (26%) among our patients seeking consultation for suspected tick-borne illness. This is a novel finding and higher than the seropositivity rates of 3–21% previously reported in the literature (13–15). There are likely many factors that may contribute to this finding. Firstly, *B. miyamotoi* is a common co-infection found in ticks (16). Given that *B. miyamotoi* is found 10 times less frequently in ticks than *B. burgdorferi* (17); we should still suspect at least 30,000 cases of *B. miyamotoi* disease in the US, compared to 300,000 presumed yearly cases of Lyme disease in the US (18). Secondly, unlike *B. burgdorferi*, *B. miyamotoi* can be transmitted via transovarial transmission, directly from adult tick to offspring, such that larval ticks can transmit infection as well as later stages (19). Thirdly, *B. miyamotoi* transmission from tick to human also occurs more quickly than *B. burgdorferi* transmission, the former occurring within 24 h of tick attachment, and the latter between 48 and 72 h (20). *B. miyamotoi* has been found in the midgut and salivary glands of both *Ixodes scapularis* and *Ixodes ricinis* ticks, likely contributing to faster transmission rates (21, 22). Lastly, it is possible that our clinical population is enriched for prior *B. miyamotoi* infection, as many patients presented with chronic symptoms and exposure to Lyme-endemic areas, but without erythema migrans rashes or positive Lyme serologic tests.

Our antibody assay detected the GlpQ antigen. GlpQ is found in other relapsing fever spirochetes throughout the world. In the United States, there are three primary species of relapsing fever spirochetes transmitted by soft ticks: *B. hermsii, B. parkeri, and B. turicatae.* Of the three, *B. hermsii* is the most common and is predominately found in the forested mountainous regions of the western United States (23). Our population was predominantly from the northeastern United States (16 of 21 *B. miyamotoi* positive patients), so it is unlikely that this finding could be due to a cross-reactivity with these soft-tick relapsing fevers. However, given that we did not obtain detailed travel histories, we cannot exclude the possibility of cross-reactivity. Furthermore, relapsing fever infection transmitted through soft ticks is thought to be rare in the United States; between the years of 1990 and 2011, only 504 cases of tick-borne relapsing fever were reported in the western United States (24).

Another notable finding from this study is that nearly all patients had not been previously tested for *B. miyamotoi* despite histories of tick exposure and subsequent symptoms and negative Lyme disease tests. Furthermore, many patients reported that their clinicians dismissed the possibility of tick-borne illness both at the onset and during the course of their illness and attributed symptoms to psychological stress. This underscores the need for more widespread clinician awareness of *B. miyamotoi* disease, especially because unlike Lyme disease, *B. miyamotoi* infection does not commonly present with an obvious pathognomonic sign, such as erythema migrans rash.

Our high seropositivity rate in this clinical sample, coupled with lack of prior testing, represents a significant public health concern. In Lyme disease, delayed diagnosis and treatment is associated with prolonged symptoms (25). The same may prove true for *B. miyamotoi* disease. Our study raises the research question of whether *B. miyamotoi* infection can lead to chronic symptoms. To decrease the risk of prolonged morbidity, patients with an acute onset of multi-system symptoms suggestive of Lyme disease or other tick-borne illnesses should also be tested for *B. miyamotoi* infection. While the PCR assay is optimal for patient assessment in acute infection, the antibody assay helps to clarify who has been previously infected; this is particularly relevant to those whose symptoms have been present for months or longer. While a positive GlpQ IgG does not confirm current infection, it strongly supports prior infection and would provide a measure of validation and relief for patients whose symptoms suggest tick-borne disease, but all laboratory tests were negative.

Our preliminary data indicate considerable overlap in the post-treatment symptom profile of patients with *B. miyamotoi* and *B. burgdorferi* antibodies. However, our patients with *B. miyamotoi* antibodies reported more sleepiness and pain than our patients with *B. burgdorferi* antibodies. Intriguingly, none of our *B. miyamotoi*-positive patients were also *B. burgdorferi*-positive on the C6 peptide ELISA. This was unexpected given prior research showing a high Lyme C6 positivity rate (91.7%) among patients with PCR-positive *B. miyamotoi* infection (26). This discrepancy may be due to the fact that our patients with chronic symptoms had all been previously treated with antibiotics for possible Lyme disease, while patients in prior studies were tested prior to treatment when they were acutely ill with active *B. miyamotoi* infection.

A limitation of this study is that we cannot verify a causal relationship between prior *B. miyamotoi* infection and chronic, non-specific symptoms in our patient sample. Because it is difficult to determine the date of infection in an illness that does not usually present with an obvious external sign, such as a rash, it is unclear whether patients' ongoing symptoms were temporally related to *B. miyamotoi* infection. This complexity is compounded by the variable disease presentation of relapsing fever borreliosis (27). Moreover, it is unknown how long *B. miyamotoi* antibodies persist after infection. Thus, to establish causality between infection and symptoms, a prospective longitudinal study following a larger sample of patients with acute *B. miyamotoi* disease is needed. Second, because our study relied on the GlpQ antibody assay rather than on a PCR assay, we cannot determine whether current infection was present at the time of our evaluation. Third, our sample size was relatively small.

Our study demonstrates that *B. miyamotoi* disease is rarely considered in the differential diagnosis of tick-borne illness. Our findings suggest that all patients presenting with symptoms indicative of a potential tick-borne illness in the absence of an erythema migrans rash should be tested for *B. miyamotoi* disease, using both PCR and antibody-based testing. Identifying *B. miyamotoi* seropositivity among patients suffering from chronic illness represents a significant finding warranting further investigation. Our findings raise the question of whether *B. miyamotoi* infection can lead to post-treatment sequelae, similar to Lyme disease. Given that *B. miyamotoi* disease is an emerging tick-borne illness, further basic science research and *in-vitro* models are needed to clarify the mechanisms and optimal treatment of *B. miyamotoi* disease (22).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by New York State Psychiatric Institute. Written informed consent to participate in research was provided by the participants or their legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SD and BF contributed to the study design and clinical consultation. SD and LM contributed to the writing of the manuscript and BF contributed to the editing of the manuscript. SD, CA, CB, EB, LM, and BF contributed to the analysis of data and CB performed the statistical analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.567350/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Risk Factors and Outcomes of Treatment Delays in Lyme Disease: A Population-Based Retrospective Cohort Study

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Background: Longer time between symptom onset and treatment of Lyme disease has been associated with poor outcomes. Reducing time-to-treatment requires knowledge of risks for treatment delays. We conducted a population-based study to evaluate factors associated with delayed treatment of Lyme disease and the relation between delayed treatment and post-treatment Lyme disease syndrome (PTLDS).

Methods: We mailed questionnaires to 5,314 individuals with a Lyme disease diagnosis or blood test followed by an antibiotic order in the medical record of a Pennsylvania health system from 2015 to 2017. Analyses were confined to 778 respondents who reported that they were treated for Lyme disease within the past 5 years and reported a rash and/or a positive blood test for Lyme disease. Time-to-treatment was calculated as the sum of two windows before and after seeking care for Lyme disease symptoms: time to first medical contact and time under care. We used logistic regression to evaluate factors associated with delayed time-to-treatment in each time window (>14 days vs. ≤14 days) and the association between total time-to-treatment (>30 days vs. ≤30 days) and PTLDS. We used inverse probability weighting to calculate estimates for the study's source population (5,314 individuals sent questionnaires).

Results: In the source population, 25% had time to first contact >14 days, 21% had time under care >14 days, and 31% had a total time-to-treatment >30 days. Being uninsured and attributing initial symptoms to something other than Lyme disease were positively associated with delayed time to first medical contact, while seeking care at an urgent care or emergency setting (vs. primary care) was negatively associated. Diagnoses between November and April, and the absence of rash were positively associated with delays. Individuals whose treatment was delayed, defined as time-to treatment >30 days had 2.26 (95% confidence interval: 1.25, 4.05) times the odds of PTLDS as those who were treated within 30 days of symptom onset.

Conclusions: In a population-based study in Pennsylvania, one-third of Lyme disease patients reported delayed treatment, which was associated with PTLDS. To improve Lyme disease outcomes, prevention efforts should aim to reduce the time before and after seeking care.

Keywords: Lyme disease, treatment delays, post-treatment Lyme disease syndrome, time-to-treatment, disparities

INTRODUCTION

Lyme disease is on the rise in the United States, with almost 30,000 confirmed and over 13,000 probable cases in 2017 (1). Delayed treatment can lead to disseminated infection and serious complications (2, 3). Longer time between symptom onset and treatment (time-to-treatment) has been associated with poor Lyme disease outcomes (4–7). Post-treatment Lyme disease syndrome (PTLDS) is characterized by persistent or recurrent symptoms, lasting 6 months or more of fatigue, musculoskeletal pain, and cognitive complaints leading to decline in physical and social functioning (3, 8). The role of time-to-treatment in PTLDS remains unknown. Timely treatment may be important in preventing PTLDS and other long-term consequences of Lyme disease. Strategies to ensure timely treatment require a better understanding of the risk factors for treatment delays.

Of the few studies of time-to-treatment in Lyme disease, most have been confined to individuals with Lyme neuroborreliosis, a neurological manifestation of disseminated Lyme disease that occurs in about 12% of Lyme disease cases (4–7, 9). These studies have reported that longer time-to-treatment is associated with poor outcomes, including persistent Lyme disease symptoms and poor quality-of-life. No studies have evaluated the role of time-to-treatment in PTLDS, a condition that occurs in an estimated 10 to 20% of Lyme disease cases (10). PTLDS is a well-defined condition that is distinct from chronic Lyme disease, a non-specific term that has been used to describe illness in individuals with Lyme disease and around which there is ongoing debate (11). The biological basis for PTLDS is not well-understood, and no evidence-based treatment has been identified (8). Thus, exploring options for prevention is critical.

Evidence-based strategies for reducing time-to-treatment of Lyme disease are lacking, in part due to limited understanding of related risk factors. Prior studies have generally measured time-to-treatment of Lyme disease as a single time period (5–7). However, the General Model of Total Patient Delay, a widely used model that describes stages of treatment delay, differentiates the time before and after a patient sees a medical professional (12). The time between symptom onset and seeing a medical professional (hereafter, "time to first medical contact") and the time while under the care of a medical professional until receiving treatment (hereafter, "time under care") involve different actors and occur in different settings. Thus, these stages may have distinct risk factors that require different approaches for promoting timely treatment.

We conducted a retrospective cohort study of time-to-treatment among a general population sample of individuals treated for Lyme disease at Geisinger, a health system in Pennsylvania, the state with the most confirmed Lyme disease cases in the United States (13). Using self-administered questionnaire data, we characterized respondents' experiences with Lyme disease symptoms, care-seeking, diagnosis, and treatment; measured risk factors for delays in time to first medical contact and time under care; and examined associations between time-to-treatment and PTLDS.

METHODS

Study Population

Participants were identified through the Geisinger electronic health record (EHR). Geisinger serves patients across 45 Pennsylvania counties. The primary care population represents the age and sex distribution of the region's population (14). We mailed questionnaires to 5,314 adult patients who met previously described EHR-based criteria for Lyme disease between 2015 and 2017 (15). Briefly, individuals had to have a Lyme disease diagnostic code (*International Classification of Diseases*, 9th Revision, code 088.81) or both a *Current Procedural Terminology* code for a Lyme disease serologic test (enzyme immunoassay or Western blot) and an antibiotic order appropriate for Lyme disease, regardless of length of treatment, within 30 days after the sample draw. Appropriate treatment was defined by the Infectious Disease Society of America's (IDSA) recommended first or second line antibiotics (3) and three antibiotics either closely related to recommended treatments or that were historical treatments (15). We excluded antibiotic orders if the diagnosis codes linked to the medication orders were for respiratory disease, since these are common diagnoses treated with the same antibiotics as Lyme disease. A $1 bill was included with the questionnaire. Non-respondents were re-sent a questionnaire 6 weeks after the original mailing. Geisinger's Institutional Review Board approved the study.

Questionnaire Development

We developed a questionnaire to measure time-to-treatment for Lyme disease and potential related factors and outcomes, informed by interviews with Lyme disease patients and physicians (16). Based on findings from this formative work, a panel of experts specializing in epidemiology, survey research, infectious disease, and rheumatology developed the questionnaire. Questions were derived from existing instruments or created *de novo* based on scientific literature.

Time-to-Treatment

Time-to-treatment was measured (in days) as the sum of two time windows: time to first medical contact and time under care. Time to first medical contact was based on response to the question, "About how long did you wait after your first symptom of Lyme disease before contacting a medical professional?" Time under care was based on response to the question, "How long was it from your first contact with a medical provider to when you were treated for Lyme disease?"

PTLDS

PTLDS was defined based on criteria developed by Aucott et al. (8), consistent with guidelines from the IDSA (3). Participants were classified as having PTLDS if they had received antibiotic treatment for Lyme disease and reported persistent symptoms and functional deficit. Respondents were classified as having persistent symptoms if they reported that one of the following symptoms had not changed, had worsened, or had newly occurred in the 6 months after completing antibiotic treatment for Lyme disease: fatigue, muscle pain, joint pain, memory changes, difficulty finding words, or difficulty focusing. Functional deficit was defined as a standardized T score <45 of the mean of the following subscales from the 36-Item Short Form Health Survey: role limitations due to physical health, energy/fatigue, emotional well-being, or role limitations due to emotional health (10, 17). Consistent with IDSA guidelines, a participant could not be classified as having PTLDS if they reported a prior diagnosis of fibromyalgia or chronic fatigue syndrome (CFS) (3).

Lyme Disease Symptoms, Care-Seeking, Diagnosis, and Treatment

The questionnaire captured the respondents' experiences related to Lyme disease symptoms, care-seeking, diagnosis, and treatment. Items related to Lyme disease symptoms included whether the respondent observed a tick bite or a rash, whether the rash was a bull's-eye rash, the constancy of symptoms, and to what condition respondents initially attributed their Lyme disease symptoms. Items related to care-seeking included specialty of the first medical professional contacted for Lyme disease symptoms, reason for contacting the medical professional, and barriers to contacting a medical professional. The questionnaire also assessed diagnosis received at the first medical visit, whether an antibiotic was prescribed, number of medical professionals seen before receiving a diagnosis of Lyme disease, and blood testing results.

Coping

Coping was assessed using the John Henry Active Coping Scale, a 12-item scale that assesses a personality pre-disposition to cope with psychosocial stressors (18). Items were summed for a total score ranging from 12 to 60, then dichotomized at the median to categorize respondents into low and high active coping groups.

Clinical and Demographic Characteristics

Through the questionnaire, we assessed history of a diagnosis prior to Lyme disease of cancer, fibromyalgia, CFS, rheumatoid arthritis, migraine, depression, and anxiety, as well as marital status, income, education, occupation, and insurance status at the time of Lyme disease diagnosis. Age and sex were obtained from the EHR.

Statistical Analysis

The goals of the analysis were to describe time-to-treatment in a population-based sample of individuals treated for Lyme disease, to identify risk factors for the two time-to-treatment delay windows, and to evaluate associations between time-to-treatment and PTLDS. Analyses were confined to respondents who self-reported a Lyme disease diagnosis within the past 5 years, completed questions related to time-to-treatment and rash, whose Lyme disease was confirmed based on self-report of a rash and/or a positive blood test for Lyme disease, who reported being prescribed antibiotics, and for whom time-to-treatment was plausible (i.e., less than their age) ($n = 778$). We used inverse probability weighting based on EHR-based characteristics available on responders and non-responders to calculate estimates for the source population of the study (the 5,314 individuals sent questionnaires).

We conducted chi-square tests to evaluate the proportion of individuals with delays in time to first medical contact and time under care by the following variables: season of diagnosis (November–April, May–October); presence of rash (yes, no); symptom attribution (Lyme disease, other condition); first medical professional contacted [primary care, urgent care, emergency department, other (e.g., inpatient or specialist)]; self-reported diagnosis of cancer, fibromyalgia, CFS, rheumatoid arthritis, migraine, depression, and anxiety (yes, no); age (18–39, 40–49, 50–59, 60–69, ≥ 70 years); sex (male, female); insurance at time of diagnosis (private insurance, Medicaid, no health insurance, Medicare); education (less than high school, high school graduate, some college, associate degree, bachelor's degree, graduate degree); and marital status (never married, separated/divorced/widowed, married or living with a partner). For each time window a delay was described as a period lasting more than 14 days. Next, we used logistic regression to evaluate factors associated with treatment delays, separately for time to first medical contact and time under care (>14 days vs. ≤14 days). All models controlled for age (continuous), sex, insurance status, rash, and season of diagnosis. Age was tested for linearity. Additional variables that demonstrated a bivariate association with the treatment delay were added to models individually. The final models retained variables that remained associated with the treatment delay using a threshold of $p < 0.05$. We used robust standard errors, calculated using the Huber–White sandwich estimator. Model diagnostics were performed to confirm the validity of multivariable models. Hosmer–Lemeshow tests and F-tests were used to assess goodness-of-fit, while scatterplots of standardized residual vs. predicted probability of outcome were used to look for influential observations (**Supplementary Material**).

We used logistic regression to evaluate the association between time-to-treatment (sum of time to first medical contact and time under care, >30 days vs. ≤30 days) and PTLDS (yes vs. no). The base model included age-centered, age-centered

squared, sex, insurance, and time-to-treatment. We evaluated the following variables for confounding: self-reported prior diagnosis of cancer, migraine, rheumatoid arthritis, depression, or anxiety; education; occupation; marital status; and coping score ($<$ median vs. $\geq$ median). Variables were retained if adding the variable to the model changed the estimate of the association between time-to-treatment and PTLDS by at least 10%. We evaluated whether depression, anxiety, rash, and coping modified the association between time-to-treatment and PTLDS by adding cross-product terms (separately for each interaction) to the model. The same model diagnostics described above were performed. Analyses were conducted using Stata 14.1 (19).

RESULTS

Demographic and Clinical Characteristics

Of the 5,314 individuals who received a questionnaire, 1,364 returned a completed questionnaire, of whom 778 met the inclusion criteria for the analysis (**Figure 1**). Because weighted analysis accounts for potential participation bias, only weighted results are described in the text; both unweighted and weighted results are presented in tables. A little less than half of the study population was female and the mean age was 51 years (**Table 1**). At the time of Lyme disease diagnosis, 78% of the study population had private health insurance, 3% were not insured, and the remaining were insured with Medicare or Medicaid. An estimated 11.5% of the study population met the criteria for PTLDS.

Time-to-Treatment

Median time-to-treatment was 13 days (**Table 2**). An estimated 31% of study population had time-to-treatment >30 days. One-quarter reported time-to-first contact with a medical professional >14 days and 21% reported time under care >14 days. Among those with total time-to-treatment >30 days, the average ratio of time to first medical contact to time under care was 1:1, with an equal contribution of time from both delay windows.

Experiences With Lyme Disease Symptoms, Care-Seeking, Diagnosis, and Treatment

Forty-six percent of the study population reported having a bull's-eye rash, and 20% reported a rash without central clearing. About one-fifth (21%) of the population attributed their initial symptoms to Lyme disease; the remaining attributed initial symptoms to flu or a virus (34%); a bug bite, allergy, or skin problem (15%); a muscle or joint strain/injury (12%); bursitis (10%); or a mix of other conditions (**Table 2**). Nearly half of the study population reported they did not immediately contact a medical professional largely because initial symptoms were not perceived to be serious.

The majority of the study population reported initially seeking care from a primary care provider (61%). Urgent care was the first contact for an estimated 25% of the population. An estimated 56% received a diagnosis of Lyme disease at their initial medical visit (**Table 2**), though 68% of the study population reported receiving antibiotic treatment at their first visit. Most diagnoses (74%) occurred between May and October.

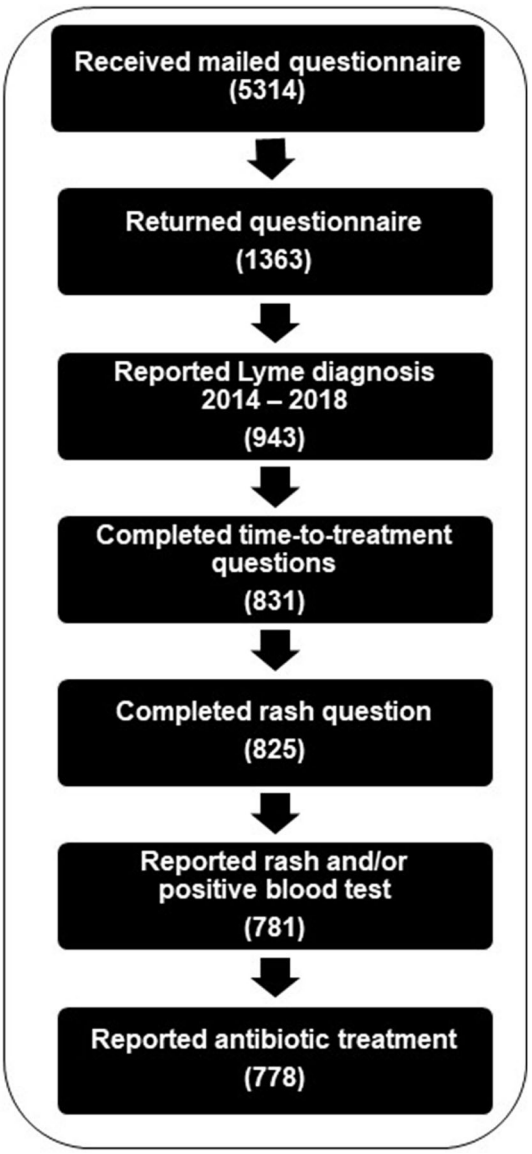

FIGURE 1 | Creation of analytic dataset of respondents to the Lyme disease time-to-treatment questionnaire, with inclusion based on responses to questionnaire items regarding date of Lyme disease diagnosis, completion of time-to-treatment and rash questions with plausible response, report of rash and/or blood test, and report of antibiotic treatment.

Factors Associated With Delayed Time to First Medical Contact

In bivariate analyses, factors associated with delayed time to first contact with a medical professional (>14 days) included younger age, no rash, Lyme disease diagnosis between November and April, misattribution of symptoms, being uninsured, first medical contact in an urgent care or emergency department setting, and self-reported diagnosis of cancer. In a model adjusted for age,

TABLE 1 | Characteristics of study population, with unweighted and weighted percentages.

	Frequencies unless otherwise noted		
	N	**Unweighted %**	**Weighted %[a]**
Total respondents	778	100	n/a
Age in years, mean		57	51
18–39	131	17	29
40–49	107	14	16
50–59	152	20	19
60–69	236	30	22
≥70	152	20	13
Female	401	52	48
Education			
Less than high school	53	7	9
High school graduate	233	30	28
Some college	141	18	20
Associate degree	84	11	11
Bachelor's degree	139	18	18
Graduate degree	128	16	15
Marital status			
Never married	83	11	16
Separated, divorced, or widowed	107	14	13
Married or living with a partner	588	76	71
Self-reported health insurance status[b]			
Medicaid (with or without Medicare)	52	7	10
Medicare only	91	12	8
No health insurance	22	3	3
Private insurance	613	79	78
Self-reported diagnoses prior to Lyme disease[c]			
Cancer	74	10	8
Fibromyalgia	26	3	3
Chronic fatigue syndrome	18	2	2
Rheumatoid arthritis	54	7	6
Migraine	88	11	11
Depression	137	18	17
Anxiety	142	18	19
PTLDS[d]			
Yes	75	10	12
No	693	89	87
Missing	10	1	1

PTLDS, post-treatment Lyme disease syndrome.
[a]Weighted by participation rates.
[b]Self-reported insurance coverage at time of Lyme diagnosis.
[c]Self-reported diagnosis by a doctor that occurred prior to Lyme disease.
[d]PTLDS based on self-reported new or persistent symptoms and functional impairment after treatment, excluding those with prior diagnosis of chronic fatigue syndrome or fibromyalgia.

sex, presence of rash, and diagnosis season, the odds of delayed time to first medical contact among those who reported being uninsured was 3.49 [95% confidence interval (CI): 1.19, 10.21] times the odds of those with private insurance. The odds of

TABLE 2 | Symptom, care-seeking, diagnostic, and treatment experiences for Lyme disease among survey respondents ($n = 778$), with unweighted and weighted proportions.

	Frequencies unless otherwise noted		
	N	**Unweighted %**	**Weighted %**
Time to treatment for Lyme disease			
Days from first symptoms to contacting a medical professional, median (range)	7 (0, 5,479)		
0–14 days	601	77	75
>14 days	177	23	25
Days from healthcare contact to treatment, median (range)	2 (0, 13,880)		
0–14 days	634	81	79
>14 days	144	19	21
Total days from first symptoms to treatment, median (range)	13 (0, 13,890)		
0–4 days	203	26	24
>4–14 days	215	28	27
>14–30 days	142	18	18
>30 days–6 months	149	19	21
>6 months	69	9	10
Experiences with Lyme disease symptoms			
Observed a tick bite	214	28	28
Reported rash[a]			
Experienced a typical bull's-eye rash	372	48	46
Experienced a rash (not bull's-eye)	163	21	20
No rash	239	31	33
Constancy of symptoms[a]			
Symptoms were constant	242	31	31
Symptoms would come and go	92	12	11
Some constant, some would come and go	375	48	51
Attributed first symptoms to Lyme disease	167	21	21
Misattributed first symptoms to other conditions[b]			
Flu or virus	251	32	34
Bug bite, allergy, or skin problem	127	16	15
Muscle or joint strain/injury	89	11	12
Arthritis or bursitis	80	10	10
Dehydration, overexertion, stress, old age	22	3	3
Other	49	6	8
Did not know	41	5	5
Experiences seeking medical care for Lyme disease symptoms			
Did not wait to contact a medical professional	421	54	51
Barriers to contacting a medical professional[b]			
Symptoms perceived to not be serious or were attributed to another cause	321	41	43
Socioeconomic barriers (e.g., cost, transportation, caregiving duties)	41	5	7
Immediate healthcare not accessible (e.g., appointments unavailable, traveling)	21	3	4

(Continued)

TABLE 2 | Continued

	Frequencies unless otherwise noted		
	N	**Unweighted %**	**Weighted %**
Reason for contacting a doctor[b]			
Suspected Lyme disease (e.g., tick bite, bull's-eye rash, previous experience)	95	12	11
New symptoms appeared	152	20	22
Symptoms did not go away	340	44	44
Symptoms got more severe	315	40	43
Symptoms interfered with work or daily tasks	175	22	27
Family or friend said to go	146	19	19
Experiences with diagnosis and treatment for Lyme disease symptoms			
First medical professional contacted about symptoms[a]			
Urgent care	190	24	25
Emergency department	85	11	12
Primary care	477	61	61
Other[c]	25	3	3
Diagnosis received at first medical visit[a]			
Lyme disease or suspected Lyme disease[d]	455	58	56
Flu or other viral infection	50	6	6
Skin rash, allergic reaction, shingles	47	6	6
Muscle or joint injury	30	4	5
Cellulitis or other skin infection	23	3	3
Insect bite	22	3	3
Arthritis	5	1	1
Other	36	5	5
None	97	12	13
Number of medical professionals seen for Lyme disease symptoms before receiving a Lyme disease diagnosis[a]			
0–1	423	54	52
2	140	18	19
≥3	91	12	13
Medical care provider who diagnosed respondent's Lyme disease[a]			
Urgent care clinic doctor	154	20	19
Emergency department doctor	72	9	9
Primary care or family doctor	432	56	55
Specialist (e.g., rheumatologist, cardiologist, neurologist, infectious disease doctor)	75	10	10
Lyme specialist	25	3	4
Self-diagnosis or other non-medical diagnosis	10	1	1
Diagnosis season[a]			
May–October	582	75	74
November–April	136	17	18
Blood testing[a]			
First test was positive	501	64	63
First test was negative, second test was positive	102	13	16
Blood tests only negative	47	6	6
Blood never tested	110	14	13
Received antibiotic treatment at first medical visit			
Yes	542	70	68
No	236	30	32
Lyme disease treatment received[a]			
1 oral antibiotic	556	71	70
1 intravenous antibiotic or 2 oral antibiotics	135	17	17
>2 antibiotics	76	10	11

aCategories do not add to 100% of sample due to missing data.
[b]Categories are not mutually exclusive.
[c] "Other" includes specialists (e.g., dermatologist) and inpatient/hospital.
[d]Respondent indicated there was no diagnosis, but blood testing was ordered.

delayed time to first medical contact among those who initially attributed their symptoms to something other than Lyme disease was 3.51 (95% CI: 1.79, 6.89) times the odds of those who initially attributed symptoms to Lyme disease (**Table 3**). Odds of delay among individuals who initially sought care in an urgent care or emergency department setting were 0.33 (95% CI: 0.17, 0.64) and 0.37 (0.17, 0.81), respectively, times the odds of those who sought care from a primary care provider. The odds of delay among who reported a rash was 0.44 (95% CI: 0.27, 0.71) times the odds among those without rash.

Factors Associated With Time Under Care

In bivariate analyses, factors associated with delayed treatment while under care of a medical professional (>14 days) included younger age; never married; unable to work/disabled; no rash; Lyme disease diagnosis between November and April; first medical contact in an emergency department or "other" setting; and self-reported diagnosis of fibromyalgia, CFS, or migraine prior to Lyme disease. In models adjusted for age, sex, and insurance status, rash was associated with nearly half the odds of delay under care (**Table 4**). The odds of the delay among those diagnosed between November and April was 2.36 (95% CI: 1.37, 4.07) times the odds of those diagnosed at other times of the year. The odds of delay among those with a diagnosis of chronic fatigue syndrome was 5.02 (95% CI: 1.79, 14.12) times the odds among those without a diagnosis.

Time-to-Treatment and PTLDS

The odds of PTLDS among those with time-to-treatment >30 days was 2.26 (95% CI: 1.25, 4.05) times the odds of those treated within 30 days, adjusting for age (centered and centered-squared), sex, and insurance status. Depression, anxiety, presence of rash, and coping did not modify the association between time-to-treatment and PTLDS.

TABLE 3 | Logistic regression analysis of factors related to delays in contacting a medical professional[a] for Lyme disease.

	Study sample ($n = 717$[b]) unweighted	Source population weighted[c]
Respondent characteristic	**Odds ratio (95% CI)**	**Odds ratio (95% CI)**
Age	0.98 (0.97, 1.00)	0.97 (0.96, 0.99)
Sex, female	0.73 (0.50, 1.08)	0.70 (0.44, 1.10)
Insurance[d]		
Privately insured	Ref	Ref
Medicaid only or with Medicare	1.03 (0.51, 2.07)	1.26 (0.61, 2.62)
No health insurance	3.09 (1.21, 7.86)	3.49 (1.19, 10.21)
Medicare only	1.50 (0.77, 2.92)	1.84 (0.92, 3.69)
Presence of rash	0.39 (0.26, 0.58)	0.44 (0.27, 0.71)
Diagnosis season		
May–October	Ref	Ref
November–April	2.20 (1.42, 3.41)	2.60 (1.60, 4.21)
Attributed first symptoms to Lyme disease		
Yes	Ref	Ref
No	2.93 (1.67, 5.14)	3.51 (1.79, 6.89)
First medical provider contacted about Lyme disease symptoms		
Primary care/family doctor	Ref	Ref
Urgent care clinic	0.38 (0.22, 0.66)	0.33 (0.17, 0.64)
Emergency department	0.49 (0.27, 0.89)	0.37 (0.17, 0.81)
Other[e]	1.48 (0.61, 3.60)	1.23 (0.44, 3.44)

[a] *Delay characterized as >14 days (vs. ≤14 days) from first symptoms of Lyme disease to contacting a medical professional, as reported by respondents.*
[b] *Data on rash, diagnosis season, and first medical provider contacted about Lyme disease symptoms missing for 61 respondents.*
[c] *Weighted by participation rates.*
[d] *Self-reported insurance coverage at time of Lyme diagnosis.*
[e] *"Other" includes specialists (e.g., dermatologist) and inpatient/hospital.*

TABLE 4 | Logistic regression analysis of factors related to delays between healthcare contact and treatment[a] for Lyme disease.

	Study sample ($n = 718$[b]) unweighted	Source population weighted[c]
Respondent characteristic	**Odds Ratio (95% CI)**	**Odds Ratio (95% CI)**
Age	0.98 (0.97, 1.00)	0.98 (0.96, 1.00)
Sex, female	0.96 (0.64, 1.43)	1.06 (0.66, 1.71)
Insurance[d]		
Privately insured	Ref	Ref
Medicaid only or with Medicare	1.43 (0.72, 2.84)	1.09 (0.48, 2.50)
No health insurance	1.13 (0.41, 3.18)	1.13 (0.40, 3.21)
Medicare only	0.51 (0.22, 1.17)	0.75 (0.25, 2.28)
Rash accompanied Lyme disease	0.52 (0.34, 0.78)	0.56 (0.34, 0.91)
Diagnosis season		
May–October	Ref	Ref
November–April	2.07 (1.32, 3.25)	2.36 (1.37, 4.07)
Chronic fatigue syndrome[e]	5.03 (1.90, 13.29)	5.02 (1.79, 14.12)

[a] *Delay characterized as >14 days (vs. ≤14 days) from first contact with a medical provider to treatment for Lyme disease, as reported by respondents.*
[b] *Data on rash and diagnosis season missing for 60 respondents.*
[c] *Weighted by participation rates.*
[d] *Self-reported insurance coverage at time of Lyme diagnosis.*
[e] *Self-reported diagnosis (yes vs. no) by a doctor that occurred prior to Lyme disease.*

DISCUSSION

In this first population-based study of time-to-treatment of Lyme disease, we characterized experiences with Lyme disease symptoms, care-seeking, diagnosis, and treatment among individuals in Pennsylvania, a state highly endemic to Lyme disease; identified common and unique factors associated with delays before and after contacting a medical professional; and evaluated long-term consequences of delayed treatment. In a novel finding, we observed that time-to-treatment was associated with PTLDS, demonstrating the potential long-term consequences of delayed treatment. Several factors—including insurance status, the presence of a rash, diagnosis season, attribution of initial symptoms to Lyme disease, the first medical provider contacted about the symptoms, and a diagnosis of chronic fatigue syndrome prior to Lyme disease—were related to treatment delays. These findings have important implications for strategies to reduce time-to-treatment in Lyme disease and the potential of these efforts to improve long-term outcomes.

We found that delayed treatment was associated with higher risk of PTLDS. Although our study is the first to evaluate time-to-treatment in relation to PTLDS, the findings are consistent with prior studies that examined persistent symptoms. Rebman et al. (11) found that in a sample of individuals with PTLDS, 45% reported time-to-treatment >30 days. Negative consequences of delayed treatment for Lyme disease have been previously reported, with longer time-to-treatment associated with persistent symptoms, poor quality-of-life, and Lyme neuroborreliosis, but none, to our knowledge, have demonstrated an association with PTLDS specifically (4–7, 20). The benefit of shorter time-to-treatment has been attributed to the prevention of pathogen dissemination, resulting from earlier eradication of the *Borrelia burgdorferi* bacterium (5). Alternative hypotheses for the benefit of early antibiotic treatment include early interruption of the immune response, which may prevent secondary autoimmune reactions (5). While the pathogenesis of PTLDS remains unknown, an autoimmune response is one of the hypothesized causes (8).

Averting treatment delays in Lyme disease may be a key strategy for preventing PTLDS and other serious complications. Prior studies of Lyme disease have defined treatment delay as the time between symptom onset and treatment, with definitions of delay ranging from >30 days to >6 weeks (4, 5, 7, 11). We found that time-to-treatment >30 days has potentially important implications for Lyme disease outcomes, as this delay was associated with more than twice the odds of PTLDS. Of concern, 31% of our study population reported time-to-treatment exceeding 30 days. Other studies have also reported a large proportion of individuals with time-to-treatment longer than 30 days (7, 11). Thus, there remains a substantial delay

in Lyme disease care that, if closed, could improve Lyme disease outcomes.

We found that the two time windows comprising time-to-treatment (both before and after contacting a medical professional) contributed equally to Lyme disease treatment delays. One prior study used similar time-to-treatment windows to evaluate individuals with Lyme neuroborreliosis, observing even longer delays than in our study, with a median time from symptom onset to first hospital contact of 20 days and a median time from first hospital contact to treatment of 24 days (4). Thus, there are opportunities to shorten time-to-treatment both before and after an infected individual engages with the healthcare system.

The absence of a rash was a strong factor in delayed treatment for Lyme disease, as it was associated with both delay windows, signifying its importance to both individual and provider behavior. The association of rash with delayed time to medical contact aligns with a prior qualitative study that revealed patients with treatment delays ruled out the possibility of Lyme disease because they did not observe a bull's-eye rash (16). Similar to our findings, past reports indicate that up to 30% of people with Lyme disease do not present with erythema migrans (21) and a subset of these individuals do not present with the characteristic bull's-eye appearance (21). On the healthcare side, misdiagnosis reportedly occurs more commonly among patients with Lyme disease that do not present with erythema migrans (22). This work suggests that efforts to reduce time-to-treatment should include educational campaigns targeting patients and healthcare providers on alternative clinical presentations of Lyme disease and erythema migrans (22).

Delays before and after contacting a medical professional were also more common for Lyme disease diagnosed between November and April compared to other times of the year. A prior study of Lyme neuroborreliosis similarly reported longer time-to-treatment when Lyme disease occurred in winter and early spring (4). This is the time of year when Lyme disease is least commonly contracted (1), thus patients and medical professionals may be less likely to attribute symptoms to Lyme disease in this time period. Though less common in these months, thousands of confirmed cases of Lyme disease are reported from November to April (1). Building awareness among patients and medical providers of the risk of Lyme disease throughout the year in endemic regions provides another opportunity for reducing time-to-treatment.

Uninsured individuals in our study were more likely to delay contacting a medical professional for their symptoms than were individuals with private insurance. This finding aligns with a prior qualitative study of treatment delays in Lyme disease, which highlighted the symptoms that individuals endured while waiting to obtain health insurance, including debilitating joint pain and dangerously high fevers (16). Treatment delays due to lack of insurance occur for a range of conditions, from myocardial infarction (23, 24) to cancer (25), and improving accessibility of health insurance is a critical goal in efforts to provide timely treatment. Considering that the costs of diagnosing and treating acute and uncomplicated Lyme disease are relatively inexpensive (26), diagnostic tests and treatment should be made accessible and affordable for those with and without health insurance.

Most participants reported initially contacting a primary care provider for their Lyme disease symptoms. However, these individuals were at greater risk of delayed treatment than individuals who sought care in an urgent care or emergency department setting. Wait times for primary care appointments can be lengthy, and many primary care clinics do not offer evening or weekend hours (27). In our study, the inability to obtain care outside of work hours or while traveling away from home, and responsibilities such as caregiving duties were noted as barriers to seeking prompt care for Lyme disease symptoms. Urgent care clinics offer an important option for individuals who might otherwise delay contacting a medical professional. Increasing use of urgent care clinics for Lyme disease symptoms may require public health campaigns to inform the general population of the importance of prompt treatment for Lyme disease.

A self-reported diagnosis of CFS prior to Lyme disease increased the odds of delay while under care. Considering the similarity in some symptoms in the two conditions, health care providers may not have initially recognized the onset of Lyme disease symptoms as a new condition, resulting in delayed treatment. Alternatively, CFS may have been later misdiagnosed as Lyme disease, or Lyme disease may have been initially misdiagnosed as CFS (28). Given the small number of individuals in our sample with CFS, these findings should be considered preliminary.

The strengths of this study include a population-based sample from a Lyme endemic state, identification of separate risk factors associated with two time windows of treatment delays that potentially require unique approaches to reducing delays, and evaluation of the association between time-to-treatment and PTLDS using guideline-based criteria that includes persistent symptoms and functional deficit. This study had some limitations. First, we did not require a positive blood test when identifying Lyme disease cases. It is possible that some study respondents did not have Lyme disease, though unlikely given the combination of EHR data—which has demonstrated utility in identifying Lyme disease cases (15)—and self-reported data to identify cases. Confining the study to individuals with a positive blood test would have excluded individuals who were promptly treated with antibiotics or tested before antibodies developed, resulting in an overestimation of time-to-treatment. Second, individuals with longer time-to-treatment or with persistent symptoms may have been more likely to respond to the questionnaire, potentially resulting in an overestimation of time-to-treatment and its association with PTLDS. To mitigate participation bias, we employed inverse probability weighting. Third, the study population was diagnosed with Lyme disease at Geisinger, a single integrated health system. However, Geisinger has more than 44 community practice sites, 12 hospital campuses, and more than 20 urgent care clinics across a large geographic region;

thus, the findings reflect the practices of Lyme disease diagnosis across a range of clinical settings. Moreover, questionnaires captured information on experiences within and outside of Geisinger. Finally, our findings may be subject to same-source bias due to the use of self-reported data for both exposures and outcomes.

CONCLUSIONS

In a population-based study of Lyme disease in Pennsylvania, treatment delays, defined as time-to-treatment >30 days, were reported by nearly one-third of individuals with Lyme disease. Delays before and after contacting a medical professional had common and unique risk factors. Delayed treatment was associated with PTLDS. To improve long-term outcomes of Lyme disease, strategies for preventing delayed treatment should aim to reduce both the time before and after contacting a medical professional.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Geisinger Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

AH, MP, and BS conceived and designed the analysis. CN performed the data analysis. AH, BS, MP, KM, AR, JA, CH, and BS contributed to the interpretation of results. All authors contributed to the writing and final review of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2020.560018/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Report of the Pathogenesis and Pathophysiology of Lyme Disease Subcommittee of the HHS Tick Borne Disease Working Group

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An understanding of the pathogenesis and pathophysiology of Lyme disease is key to the ultimate care of patients with Lyme disease. To better understand the various mechanisms underlying the infection caused by *Borrelia burgdorferi*, the Pathogenesis and Pathophysiology of Lyme Disease Subcommittee was formed to review what is currently known about the pathogenesis and pathophysiology of Lyme disease, from its inception, but also especially about its ability to persist in the host. To that end, the authors of this report were assembled to update our knowledge about the infectious process, identify the gaps that exist in our understanding of the process, and provide recommendations as to how to best approach solutions that could lead to a better means to manage patients with persistent Lyme disease.

Keywords: Lyme disease, pathogenesis, pathophysiology, health and human services, tick borne disease working group

INTRODUCTION

This Report focuses on the pathogenesis and pathophysiology of Lyme disease. There are other HHS TBDWG subcommittee reports that instead focus on clinical aspects of Lyme disease, and other tick-borne diseases, including issues related to the treatment of these diseases, that are posted on the HHS TBDWG website. Here we summarize presentations by subcommittee members, as well as those of several other, invited investigators. It is recognized that there are many other important contributions by notable investigators in the area of pathogenesis and pathophysiology of Lyme disease and other tick-borne diseases that have not been included here, due to time-limitations for the subcommittee.

BACKGROUND

An understanding of the pathogenesis and pathophysiology of Lyme disease is key to the ultimate care of patients with Lyme disease. To better understand the various mechanisms underlying the infection caused by *Borrelia burgdorferi*, the Pathogenesis and Pathophysiology of Lyme Disease Subcommittee was formed to review what is currently known about the pathogenesis and pathophysiology of Lyme disease, from its inception, but also especially about *B. burgdorferi's* ability to persist in the host. To that end, the authors of this report were assembled to update our knowledge about the infectious process, identify the gaps that exist in our understanding of the process (**Figure 1**), and provide recommendations as to how to best approach solutions that could lead to a better means to manage patients with persistent Lyme disease.

It has been established that the major causative organism of Lyme disease, *B. burgdorferi*, can persist in a number of animal models and human case studies following infection and treatment with a "standard" course of antibiotics (1–4). However, it is still unclear whether human patients with ongoing symptoms associated with Lyme disease continue to have an active infection following completion of what seems as appropriate antibiotic therapy. Thus, the extent to which unresolved infection, incomplete clearance of borrelial antigens, and/or autoimmunity contribute to persistent Lyme disease symptoms is unclear (5, 6).

To better understand the pathogenesis and pathophysiology of Lyme disease, the progression of *B. burgdorferi* from its reservoir in the *Ixodes* tick to transmission into the vertebrate host and to its localization and persistence in neural and other tissues are key steps toward finding means to resolve the infection. The following are descriptions of some of what is known about these various factors of the pathogenesis and pathophysiology of Lyme disease.

Transmission and Dissemination of *B. burgdorferi* in the Vertebrate Host

In the midgut of a molted, unfed tick, *B. burgdorferi*'s survival in a dormant state requires only a small amount of energy, because little to no bacterial replication occurs (7). Outer surface proteins (Osps) facilitate the pathogen's adhesion to midgut tissue. A tick's ingestion of blood provides *B. burgdorferi* with copious nutrition, resulting in rapid bacterial replication. In turn, *B. burgdorferi* stops producing tick-specific adhesins and starts producing OspC and other factors required for transmission of the pathogen to vertebrates (8). After initiation of a blood meal, the infected tick's midgut swells, and the junctions between midgut cells become thinner. *Borrelia burgdorferi* then penetrates those junctions and enters the tick's salivary glands and salivary ducts, thereby setting the stage for its transmission to a vertebrate *via* tick bite. Upon injection into the vertebrate host, the bacteria adhere to tissues and replicate at the bite site (8, 9). Dissemination of *B. burgdorferi* throughout the vertebrate host involves migration through tissues, as well as transport *via* the bloodstream, resulting in a brief period of bacteremia.

There are a number of questions meriting additional investigation, including processes occurring inside the tick, as well as the processes of initial entry and dissemination, such as the following:

- How does *B. burgdorferi* sense its location in the tick-mammal infectious cycle, then use that information to regulate production of its proteins?
- What are the signals that "tell" *B. burgdorferi* that a vector tick is feeding and that it is time to transmit out of the tick?
- How does *B. burgdorferi* get into the tick's salivary glands and salivary ducts?
- How does *B. burgdorferi* control production of host-specific proteins?
- When bacteria adhere to host tissues at the tick's bite site and then replicate, to what kinds of tissues do they adhere? What types of proteins is *B. burgdorferi* making to facilitate adherence?
- Upon infection of a human, how does *B. burgdorferi* spread? It is known to migrate through skin and other solid tissue, but does it go through the lymphatic system or attach to nerve endings? Does it localize in sensory ganglia? What is the role of adhesins in dissemination throughout the vertebrate host? Are there particular host tissues that attract *B. burgdorferi*?

Gene Regulation of *B. burgdorferi* During Colonization, Dissemination, and Tissue-Specific Infection in Mice

Borrelia burgdorferi can sense whether it is located in a tick or mammal and adapt its response to environmental signals, such as temperature, pH, oxygen levels, carbon dioxide levels, nutrient availability, and reactive oxygen species (7). The rate of bacterial replication has effects on expression levels of numerous infection-associated genes and proteins. Carbon dioxide is important in determining the virulence of *B. burgdorferi* in mice. Borrelial oxidative stress regulator plays a pivotal role in establishing mammalian infection. *B. burgdorferi* can grow and survive without iron; genes generate an oxidative stress response that is involved in the transport of manganese and other metals within *B. burgdorferi*-infected mice. The use of bioluminescent *borrelia* as a tool for studies in mice allows visualization of the kinetics of infection with different strains of the pathogen and enables real-time evaluation of gene expression in the skin, heart, and joints of a mammal infected with *B. burgdorferi*. Notably, localized infection with *B. burgdorferi* becomes more difficult to detect as the pathogen disseminates throughout the mouse. An important gap in knowledge is that it is yet to be determined which genes are required for dissemination of *B. burgdorferi* and its colonization of tissues during later stages of infection (10).

Role of the Immune System in Response to *B. burgdorferi* Infection

Borrelia burgdorferi establishes persistent and non-resolving infections in fully immunocompetent mice, strongly suggesting that the bacteria have developed multiple and likely complex immune evasion strategies (9, 11). Both innate and adaptive

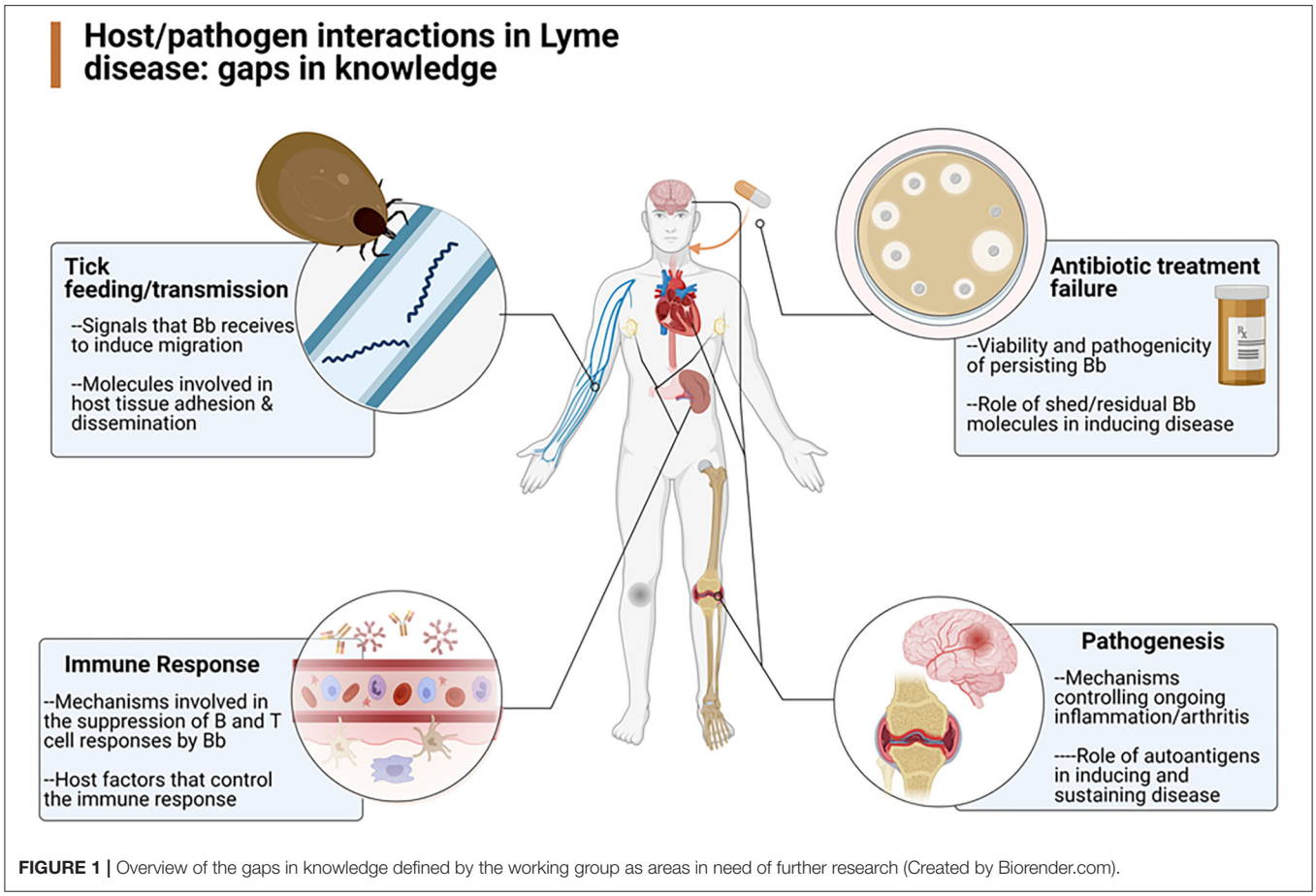

FIGURE 1 | Overview of the gaps in knowledge defined by the working group as areas in need of further research (Created by Biorender.com).

immune responses control *B. burgdorferi* in these hosts [reviewed in (12)]. These species rarely, and only transiently, develop clinical manifestations of disease, without an obvious correlation between the tissue-loads of *B. burgdorferi* and clinical manifestations, except in severely immunocompromised mice, for example those that lack T and B cells (SCID mice), or the ability to activate innate immune effectors because of deletions in the toll-like receptor (TLR) 2 or TLR adaptor protein MyD88 (13–15). MyD88-mediated innate immune responses appear to be particularly critical during earliest stages during the establishment of infection (16). Immunoglobulin (Ig) G but not IgM antibodies control *B. burgdorferi* tissue loads, but cannot clear the infection, even when the antibodies are able to passively protect from infection of a new host. IgG acts at least in part through complement-mediated opsonization of the bacteria for subsequent update by macrophage and granulocytes. Data suggest that *B. burgdorferi* suppresses effective innate and adaptive immunity (9, 11); therefore, the immune system is key to understanding persistence of Lyme disease.

B cell responses in these reservoir species are characterized by a lack of continued antibody affinity maturation and the development of long-lived responses due to the rapid collapse of germinal centers. *Borrelia burgdorferi* infection appears to suppress the adaptive immune response, as indicated by the reduced immune response to influenza vaccine in mice infected with *B. burgdorferi* (17). Ongoing work suggests that *B. burgdorferi* also prevents CD4 T cells from mounting an effective immune response to infection, potentially dysregulating effector immune responses in tissues and failing to suppress persistent infection of the host. Data were presented to support the hypothesis that *B. burgdorferi* suppresses and subverts adaptive humoral and cellular immunity to itself and to other antigens. Identifying host immune targets of *Borrelia*-mediated immune suppression might result in the development of approaches that enhance host immunity to this pathogen in a manner similar to strategies that are currently being explored in anti-tumor immunity.

Notably, mice, as reservoir hosts, never clear *B. burgdorferi* infection without antibiotic treatment; humans and non-human primates appear to harbor low-level, persistent *B. burgdorferi* infection as well (18–20). Persistence appears to be a function of active immune suppression and immune evasion tactics. An assay that was developed to detect antibody responses to five antigens of *B. burgdorferi* infection following antibiotic treatment (21) showed that most rhesus macaques infected with *B. burgdorferi* generated responses to most of the antigens, but two showed no specific antibody responses to these antigens (22). In one study in humans, patients who returned to health after

antibiotic treatment generated the strongest antibody response (23), reflected by the percentage of plasmablasts that circulated in the blood (24), while those with persisting symptoms had weak responses to antigens or had an anti-oligopeptide permease A2 antibody titer that did not decline. The reasons why some patients develop a good antibody response remain to be determined but might be attributed to host immune factor differences or to differences in the infecting strains of *B. burgdorferi*.

Further studies of immune function in non-human primates previously vaccinated with *B. burgdorferi* found that IgM-producing cells were more frequent and persistent in *B. burgdorferi*-infected primates, results similar to those observed in human patients with persistent Lyme disease as well as in mice. Memory B cells and plasmablasts were reduced in *B. burgdorferi*-infected, unvaccinated macaques compared with vaccinated macaques; whereas CD4 T-cell memory populations appeared similar among groups, activation of T cells was somewhat dampened in the *B. burgdorferi*-infected primates. Areas for future research include determining how long *B. burgdorferi*-induced immune suppression lasts and the impact of persistent infection on effectiveness of vaccines.

Chemotaxis, Motility, and Immune Evasion as Key Factors in *B. burgdorferi* Spirochete Persistence

Most spirochetes use flagellin proteins as "motors," with which they move back and forth. This movement can be tracked in real time in mice with the use of multiphoton/confocal microscopy and fluorescently labeled *B. burgdorferi*. Ongoing imaging analysis revealed that the number of spirochetes peaked around 7–10 days after infection (12). This peak was followed by a dramatic drop in spirochete numbers, where they persisted for the duration of the experiment. Spirochetes often tend to reside in the dermis. Of the various resident immune response cells, Langerhans cells were not as effective as macrophages, or other dendritic cells or neutrophils in phagocytosing the bacteria, as spirochetes move up to 80 times faster than any of these immune response cells (12). Neutrophils responded the fastest, but after a certain point, they stop responding, leaving a number of viable spirochetes. There remains a gap in the understanding of the signals involved in this apparent suppression of neutrophil responses. Interleukin 10, the most well-characterized and immunosuppressive cytokine known, is induced early by *B. burgdorferi* to control the innate immune response (25). The innate immune response is important for controlling early infection, independent of the presence of T and B cells. *B. burgdorferi* stimulates several pattern recognition receptors of the innate immune response, inducing pro-inflammatory cytokines (26). Evasion of the innate immune response is accomplished also by multiple complement-binding proteins expressed by *B. burgdorferi* (27), dampening the initial response, as well as IgG-mediated effort functions. Greater understanding is still needed regarding the different roles of the innate and adaptive arms of the immune system in regulating immunity to the spirochetes.

Role of CD47 and the Immune Response to *B. burgdorferi*

Up-regulation of CD47, a relatively conserved "marker of self," is a newly discovered mechanism of immune evasion by *B. burgdorferi*. When CD47 binds to signal regulatory protein alpha (SIRP-alpha), there is an inhibition of phagocytosis of those cells, by macrophages. Anti-CD47 antibodies are currently under evaluation in clinical trials for cancer treatment (28, 29). It is hypothesized that *B. burgdorferi* (among other pathogens) can mimic CD47 and thus prevent macrophages from destroying *Borrelia via* phagocytosis (30). Imaging studies of the immune response to *B. burgdorferi* shows that macrophages can send out a "lasso" that wraps around *B. burgdorferi* spirochetes and draws them into the macrophage, usually the first step in the process of phagocytosis. In a few cases, the spirochetes reside in the macrophage but never appear to reach the lysosome, which is where bacterial destruction usually occurs. In donor sera, the addition of the SIRP-alpha binding domains of its receptor CV1G4 *in vitro* can result in increased phagocytosis, presumably by blocking serum-derived SIRP-alpha to CD47-like molecules on the spirochete (31). To understand why the response is more efficient in some settings, the genetic sequences of CD47 and SIRP-alpha were studied showing that SIRP-alpha is highly polymorphic (31). While a number of polymorphisms of CD47 do exist, they are infrequent in humans. Evolutionarily, there has been long-term balancing selection, which ensures that proteins that are vital to the immune response are maintained with maximum diversity, perhaps because the pathogens see some types of SIRP-alpha as beneficial to them. By using mass spectrometry and CV1G4 as a binding partner, a *Borrelia* protein was identified as a CD47-like anti-phagocytic signal. In the absence of this protein, macrophages were more effective in clearing cells. Whether *B. burgdorferi* can survive by inhibiting phagolysosome fusion, as is the case with a number of other known persistent pathogens (32), is currently unknown.

VlsE Protein-Mediated Immune Evasion

VlsE is a surface-expressed protein able to undergo extensive antigenic variation (33–35). Its expression and ability to undergo antigenic variation is required for *B. burgdorferi* survival and persistence in the presence of a host humoral antibody response targeted against VlsE (36), but also against other surface proteins. A longstanding question has been how *B. burgdorferi* immune escape is accomplished through sequence variation of this single lipoprotein can accomplish immune escape, despite the presence of a substantial number of additional antigens residing on the bacterial surface. A function for VlsE other than its antigenic variation, and thus constant evasion from the humoral antibody response, is not currently known to exist. Although other forms of immune evasion have been proposed, antigenic variation occurs even in antibody-deficient severe combined immunodeficient mice. Among the several models that have been suggested, one scenario proposes that VlsE may act as a shield to obscure the epitopes of other surface antigens (37).

One example of this is the immunogenic Arp protein of *B. burgdorferi*, which is responsible for joint inflammation during

infection. Despite Arp eliciting a strong humoral response, antibodies fail to clear the infection. Subsequent studies revealed that VlsE seems to prevent binding of Arp-specific antibodies to the surface of *B. burgdorferi*, thereby providing a possible explanation for the failure of Arp antisera to clear the infection. However, other surface-expressed proteins of *B. burgdorferi* do not seem to be blocked by expression of VlsE, and Arp remains highly immunogenic. Thus, VlsE does not appear to be a universal protector of all *B. burgdorferi* cell surface antigens. Therefore, other, as-of-yet-unknown mechanisms of immune evasion from antibody-mediated *Borrelia* clearance may exist.

Evidence That Persisting *B. burgdorferi* Are Metabolically Active and Induce Host Gene Expressions

Evidence now exists, from the results of experiments in both murine and non-human primate models, that persisting *B. burgdorferi* can be metabolically active, expressing certain bacterial genes and inducing gene expression changes in the infected host, despite being non-culturable following antibiotic treatment (22, 37–39). In one model, the spirochetes localized to the dura mater of the brain, associated with large-scale changes in gene expression of pro-inflammatory cytokines and chemokines (40, 41). Although there was no evidence of direct infection of the brain itself in this model, certain brain tissues expressed genes related to interferon signaling pathways. Gene expression of other brain functions—for example, glutamate receptors—have not yet been studied. These results, then, provide support for the hypothesis that it is persisting infection that is the cause of persisting symptoms in patients with persistent Lyme disease.

One of the greatest challenges is to actually find means to intervene in the infectious process, especially if no specific markers can be found because of low infectious load or if organisms are in locations other than blood, urine, or cerebrospinal fluid normally used for diagnosis of Lyme disease. Whether different antibiotic regimens can be found to eliminate the persistent state is another challenge that it is hoped can be met with additional targeted research.

Role of *B. burgdorferi* in the Pathogenesis and Persistence of Lyme Arthritis

Borrelia burgdorferi peptidoglycan, the primary component of the bacterial cell wall, has a unique composition and plays an important role in bacterial physiology and host immune responses. *Borrelia burgdorferi* lack the molecular machinery required for recycling of peptidoglycan during cell replication, and the bacteria shed copious amounts of peptidoglycan fragments (42). These fragments are recognized by a host pathogen recognition receptor, NOD2, and cells stimulated with peptidoglycan fragments produce high levels of pro-inflammatory cytokines. Synovial fluid from some human patients with Lyme arthritis, many of whom had received 1–3 months of antibiotic therapy, had high levels of detectible peptidoglycan, as well as anti-peptidoglycan antibodies, despite a lack of any evidence of ongoing infection after antibiotic therapy (42). Thus, it appears that *B. burgdorferi* peptidoglycan might be a persistent antigen in Lyme arthritis (12). Ongoing research is being conducted to determine whether *B. burgdorferi* peptidoglycan plays a role in the pathogenesis and pathophysiology of neuroborreliosis or of persistent Lyme disease other than previously treated Lyme arthritis.

Approximately 60% of untreated individuals with Lyme disease in the United States develop Lyme arthritis. Although most patients with Lyme arthritis respond favorably to 1–3 months of antibiotic therapy, 10–20% of patients have persistent arthritis after treatment (43). A number of genetic and environmental factors contribute to persistent Lyme arthritis, such as infection by certain arthritogenic strains of *B. burgdorferi,* retained spirochetal antigens (for example, peptidoglycans), genetic risk factors, and evidence of prior joint trauma (43, 44). As in rheumatoid arthritis, the prototypical autoimmune joint disease, Lyme arthritis is frequently accompanied by autoimmune T- and B-cell responses to self-antigens (44). These unresolved inflammatory and autoimmune responses may contribute to ongoing arthritis, despite months of antibiotic therapy. Consistent with this hypothesis, nearly all patients with persistent Lyme arthritis experience resolution of arthritis when treated with immunosuppressive drugs, including non-steroidal anti-inflammatory drugs, corticosteroids, and other antirheumatic drugs, such as methotrexate or tumor necrosis factor-alpha inhibitors. Cellular analysis of the arthritic joint has shown that large numbers of IFN-gamma-positive lymphocytes are present in inflamed tissue and surrounding fluid (45). Synovial fibroblasts, the most abundant cell type in synovial tissue, show evidence of immune activation and express major histocompatibility complex (MHC) class II molecules and other immune factors associated with inflammation and lymphocyte activation (44, 45).

Several self-peptides are immunogenic in Lyme disease patients, so there seems to be a breakdown in immune tolerance to self during *B. burgdorferi* infection. Autoimmune B cell responses (but not T cell responses) can be detected early in infection in patients with erythema migrans, but these early autoimmune responses appear to be self-limiting and non-pathogenic. T cell autoimmunity accompanies B cell autoimmunity later in disease, such as during Lyme arthritis. In late-stage disease, Lyme-disease-associated autoantibodies correlate with clinical features of arthritis, suggesting that autoimmunity in Lyme disease may become pathogenic over time. Lyme arthritis progresses from early invasion of synovial tissue to early inflammatory responses to later inflammatory responses, and then to late tissue repair and wound healing (44, 45). The role of infection as an autoimmune trigger in Lyme disease is poorly understood, leading to the following questions:

- What are the mechanisms by which *B. burgdorferi* infection causes ongoing arthritic joint disease in a subset of patients?
- Are ongoing disease symptoms caused by the presence of *Borrelia* antigens (such as peptidoglycans) rather than active infection and, if so, why are they not cleared from the host?

- Does *Borrelia* infection trigger autoimmune responses in infected individuals and are these autoimmune responses pathogenic in some patients?

Questions also remain regarding the role of immunosuppressive treatments vs. differing antibiotic treatment regimens for persistent Lyme arthritis, if peptidoglycan is an inflammatory agent and persists despite 1–3 months of antibiotic therapy. Patients who have persistent Lyme arthritis may represent a different condition than do people with other Lyme disease syndromes.

Whereas, prompt treatment of early Lyme disease, using antibiotics with differing mechanisms of action, is usually effective in prevention of persistence of *B. burgdorferi* and persistent Lyme disease, similar antibiotic treatments for persistent *B. burgdorferi* in animal models and in patients with persistent Lyme disease appear to be ineffective. The reasons for this difference are unclear, but may be due to a number of possible mechanisms:

- The bacteria may be dormant or incapable of replication, yet there may be the presence of residual antigens or the periodic release of antigens, to which the host responds to produce the symptoms associated with persistent Lyme disease.
- The bacteria may be entrenched in areas either inaccessible to certain classes of antibiotics (for example, poorly vascularized connective tissue, intracellular compartments), or higher doses of antibiotics are needed to achieve levels that impede metabolic activity.
- The bacteria may become antibiotic-tolerant, requiring repeated courses of antibiotic treatment, combinations of antibiotics, or periods of treatment alternating with periods of no treatment.

There are indications that certain treatment regimens (for example, tetracycline instead of doxycycline, the combination of a macrolide antibiotic and an alkalinizing agent) are effective in treating the persistent state if given over longer durations of time rather than the usual 2–4-week periods. There is ongoing research as well, some in the discovery phase, using novel compounds to treat persisting organisms. There is also some indication that the intestinal microbiota may play an important role in the persistence or ability to eradicate persisting organisms.

SUMMARY

The results of studies into the pathogenesis and pathophysiology of Lyme disease, with the focus on the persistent state of the causative organism, *B. burgdorferi*, have begun to elucidate the mechanisms underlying the process by which the persistent state occurs. However, important gaps exist into how the process develops, from the organism's existence in the *Ixodes* tick, to its entry into the host, to its effects on the immune system, to its distribution and ability to persist in certain tissues, to its ability to persist despite innate and other host immune system responses, and to its ability to persist despite certain antibiotic treatments. But there is reason for optimism that additional research into the pathogenetic and pathophysiologic mechanisms will lead to a better understanding of the processes involved and ultimately to a better means of preventing and treating patients with persistent Lyme disease.

AUTHOR'S NOTE

This report is a condensed version of the full report that appears on the HHS TBDWG website, and has the permission of the HHS designated officer assigned to this working group.

AUTHOR CONTRIBUTIONS

All authors contributed to the discussion and writing of the Report.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Recent Progress in Lyme Disease and Remaining Challenges

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Lyme disease (also known as Lyme borreliosis) is the most common vector-borne disease in the United States with an estimated 476,000 cases per year. While historically, the long-term impact of Lyme disease on patients has been controversial, mounting evidence supports the idea that a substantial number of patients experience persistent symptoms following treatment. The research community has largely lacked the necessary funding to properly advance the scientific and clinical understanding of the disease, or to develop and evaluate innovative approaches for prevention, diagnosis, and treatment. Given the many outstanding questions raised into the diagnosis, clinical presentation and treatment of Lyme disease, and the underlying molecular mechanisms that trigger persistent disease, there is an urgent need for more support. This review article summarizes progress over the past 5 years in our understanding of Lyme and tick-borne diseases in the United States and highlights remaining challenges.

Keywords: Lyme disease, pathogenesis, diagnosis, treatment, prevention, field building, PTLD, vaccine

INTRODUCTION

According to the Center for Disease Control and Prevention (CDC), the number of vector-borne diseases reported to the National Notifiable Diseases Surveillance System (NNDSS) between 2004 and 2016 reached a total of 642,602 cases. Of these, tick-borne diseases (TBDs) accounted for 77% (491,671 cases) of reported cases with the total number of cases doubling in 13 years. The pace of emergence of new tick-borne disease cases increased not only for Lyme disease (LD), the most predominant TBD with 82% of cases, but also for spotted fever rickettsiosis, babesiosis, anaplasmosis, and Powassan disease (1). In this review, we highlight the major scientific advances made primarily in the field of LD research in the United States (USA).

LD, also known as Lyme borreliosis, is a growing health problem in the USA. LD is caused by pathogenic species in the *Borreliella* genus (for the relationship with the *Borrelia* genus, see section Genomic Insights From Borreliaceae Lineages). These spirochetal bacteria are transmitted from vertebrate reservoirs to human hosts through bites from infected *Ixodes spp.* ticks. *Borreliella burgdorferi (B. burgdorferi,* hereafter *Bb)* is the most common agent of LD in the USA (1). The CDC recently estimated ∼476,000 clinician diagnosed cases of LD every year in the USA based on insurance claims data from 2010 to 2018 (2), a significant increase from their previous estimate of ∼329,000 annual cases using similar methods to generate data from 2005 to 2010 (3). If untreated, infection with *Bb* can lead to health problems affecting the skin, joints, nervous system, or less commonly, the heart (4). While most individuals return to health following antibiotic treatment for LD, others go on to experience chronic health problems that can last months to years. One well-defined clinical subgroup of LD patients who experience ongoing symptoms following treatment is Post-treatment Lyme disease (PTLD) (see section 2.3.2 PTLD). Medical costs related to LD and PTLD are estimated to be between $712M−1.3B each year in the USA (5). The causes of PTLD are not yet well-understood but are an active area of research due to their critical importance to advancing therapy development and effective treatment for this patient population. The two most salient hypotheses for etiology of PTLD include persistence of infection or antigenic debris, persistence of inappropriate immune activation and inflammation, or some combination of these (see section Pathogenesis below). The research community has largely lacked the necessary funding to properly advance scientific and clinical understanding of LD and its sequelae, and to develop and evaluate new approaches for prevention, diagnosis, and treatment. The annual NIH investment in LD research so far has been small compared to many other infectious diseases (see **Table 1**) (6).

Considering the rapid growth in prevalence of LD and the risk for significant long-term health consequences of those infected (7), a multifaceted effort is needed to create better prevention practices, diagnostics, and treatments, along with advancing basic science about ticks, tick-borne pathogens, and the pathophysiology of LD. In this review, we summarize key advances in each of these areas over the past 5 years and identify challenges and opportunities for the field. We aim to highlight many important studies, but due to space constraints, we are not able to discuss all relevant publications. Where available, we also reference more in-depth review articles on specific topics. While LD is a global public health concern across the Northern Hemisphere, this review article is largely USA-centric in terms of manuscripts discussed since aspects of LD vary geographically, including the primary causative agents and their vectors; pathogenicity and common disease manifestations; and the prevalence and incidence of disease.

TABLE 1 | NIH support for LD research is currently low compared to other infectious diseases.

Disease	NIH Funding FY 2018 (in millions)[a]	USA reported cases in 2018	Funding per reported case in 2018
HIV/AIDS	$2,995	36,400[b]	$82,280
Malaria	$202	∼2,000[c]	∼$101,000
West Nile Virus	$36	2,647[d]	$13,600
Lyme Disease	$30	∼33,666[e] (∼476,000 estimated cases)[g]	∼$891 ($63 per estimated case)
Tuberculosis	$403	9,029[f]	$44,634

For purposes of consistency and comparison across diseases, the table uses funding per case based on the number of reported cases. A difference sometimes exists between the reported number of cases per year and estimates of the actual incidence in some infectious diseases. For LD, the difference is more than 10-fold. For example, the number of reported cases in the USA in 2018 is ∼34 k while the estimated number of annual cases is ∼476 k (g). Therefore, the research investment by the NIH for LD is around $63 per new estimated case in 2018. This table is adapted and updated from a version in the Tick-borne Diseases Working Group (TBDWG) report to Congress (6).

[a] *https://report.nih.gov/categorical_spending.aspx*

[b] *https://www.hiv.gov/hiv-basics/overview/data-and-trends/statistics*

[c] *https://www.cdc.gov/parasites/malaria/index.html*

[d] *https://www.cdc.gov/westnile/statsmaps/cumMapsData.html*

[e] *https://wonder.cdc.gov/nndss/static/2018/annual/2018-table2i.html*

[f] *https://www.cdc.gov/mmwr/volumes/68/wr/mm6811a2.htm*

[g] *https://www.cdc.gov/lyme/stats/humancases.html*

CLINICAL AND TRANSLATIONAL MEDICINE

Next, we review progress in the diagnosis and treatment of LD, including emerging diagnostic assays and novel therapies. We also describe 2-well-defined subgroups of patients with post-treatment sequelae, including those with antibiotic-refractory Lyme arthritis (LA) and PTLD.

Diagnosis

The diagnosis of LD can be a complex task for the provider because, outside of the erythema migrans (EM) lesion of early LD, diagnosis relies on non-specific clinical signs and symptoms that may or may not be supported by laboratory evidence. A prospective study evaluated the ability of emergency room (ER) physicians across 5 hospitals in endemic areas in the Northeast of the USA to accurately discriminate between LD (early disseminated or late) and non-LD using clinical judgment alone, prior to the receipt of laboratory evidence. Among

1,021 children being evaluated for LD (based on presence of one or more EM lesions or Lyme serology tests ordered and compatible symptoms) and enrolled in the study between 2015 and 2017, clinician suspicion of LD in the ER setting was found to be minimally accurate compared to diagnoses supported by laboratory evidence. Twelve percent of patients whom the treating clinician deemed to be unlikely to have LD, actually had LD. Thirty-one percent of patients whom the clinician deemed very likely to have LD, actually did not have LD (8). A true case of early disseminated or late LD was defined in this study as those with compatible symptoms and positive two-tier serology per guidelines, the limitations of which are reviewed below. The accuracy of clinician assessment of patients presenting with a single EM lesion ($n = 42$) was not assessed. The challenges of discriminating between an EM lesion of LD and a non-EM lesion are included below, along with a description of a novel imaging tool that may aid clinician assessment of this sign of LD (9).

Many patients struggle with getting a timely diagnosis and treatment for LD. Around 40% of patients diagnosed with LD have signs and symptoms associated with disseminated or late LD, indicating that delayed diagnosis and treatment are a common occurrence (10). In a recent population-based study of 778 patients surveyed in Pennsylvania who were treated for LD in the past 5 years, 31% had a time to treatment >30 days and 10% had time to treatment >6 months, where time to treatment is defined as the sum of time to first medical contact and time under care until receiving treatment (11). A qualitative study of 26 patients treated for LD in Pennsylvania suggests that patient appraisal of their own signs and symptoms plays a role in delayed treatment, specifically the misattribution of non-specific symptoms, the intermittent nature of symptoms and the lack of a "bull's-eye rash," which is commonly misunderstood to be the only representative skin lesion of LD (12). High rates of initial delayed or misdiagnosis is also commonly reported by LD patients that meet the PTLDS case definition (13) or those with chronic symptoms more broadly (14).

The consequences of the diagnostic challenges in LD are potentially significant for patients and may lead to missed or delayed diagnosis and exposure to inappropriate or inadequate treatment. Next, we review some recent findings and the current challenges related to the diagnosis of LD.

Exposure to Ticks

The collection of tick exposure history from patients suspected of having LD lacks sensitivity because ticks are stealth biters. They are able to avoid detection by human hosts during feeding. Many people diagnosed with LD have no recollection of being bitten by a tick (15). While the major endemic regions in the USA are the Northeast (16), mid-Atlantic and upper Midwest states, *Ixodes* spp. ticks capable of carrying LD pathogens are found in many states. A recent citizen-science based effort to collect ticks submitted by volunteers from across the USA identified ticks capable of carrying *Borreliella* species in 35 states (17). In California, where LD is not considered endemic, infected *Ixodes spp.* ticks have been found in 42 counties (72%) according to surveillance data (18).

Serological Testing

Two-tiered serological testing is widely used to support the diagnosis of LD. The two-tiered testing algorithm consists of a first-tier enzyme immunoassay (EIA) or ELISA, and for samples that are positive or equivocal (borderline) on the first tier, a second tier immunoblot is performed. Using the CDC's algorithm, the immunoblot is positive if at least 2 of 3 bands are present on the IgM immunoblot within 30 days of symptom onset or 5–10 bands are present on the IgG immunoblot at any time (19). A modified two-tiered testing algorithm was approved by the CDC in 2019, which uses a first-tier ELISA, and instead of the confirmatory immunoblot, uses 1 or 2 additional ELISAs that target different antigens than the first-tier ELISA (20).

Serology presents several challenges for accurate diagnosis. The human body takes time to generate anti-*Bb* antibodies, so serological testing is not sensitive during early infection (21–24)—the period when treatment is most likely to succeed. For patients with disseminated LD or later manifestations such as late Lyme arthritis, serological testing has improved performance compared to early disease (22, 25, 26). However, current tests also lack sensitivity following antibiotic treatment of acute LD, as seroconversion occurs less frequently (21, 23, 27–30). Rebman et al. ran two-tier serology on acute and convalescent sera samples collected from 104 patients with clinician-diagnosed EM rash and 21 days of antibiotic treatment. They observed 41 (39.4%) of these patients were seronegative at both the acute and convalescent time points; only 7 (6.7%) patients were observed to have IgG seroconversion at either time point (29). Seroconversion was also rare in samples from the Lyme Disease Biobank, where only 3 of 83 samples (3.6%) from patients with EM > 5 cm seroconverted at the convalescent draw (2–3 months after the acute draw) (23). Other widely known limitations of serological tests are that they are unable to distinguish between a prior exposure to *Bb* and an active infection, may cross-react with non-*Bb* antibodies, are subject to variable results depending the selection of antigens used in the first-tier test (31) and some assays, especially the Western immunoblot, require interpretation that may introduce bias (32, 33).

For patients with an EM > 5 cm in an endemic area with a history of tick exposure, a clinical diagnosis is sufficient (34). Testing is not indicated in these patients, and the serologic tests would likely be negative due to lack of antibody development in early disease. For patients with early LD presenting without EM, diagnosis is incredibly challenging.

Signs and Symptoms

If untreated, a patient with a *Bb* infection may go through several stages of LD, with different signs and symptoms at each stage [reviewed in (35)]. In most people, the first stage of LD begins with "flu-like" symptoms and an EM lesion. LD is known as the "great imitator" because symptoms are varied and often overlap with common health complaints, sometimes making early diagnosis more difficult (36, 37). The most common symptoms of early LD are fever, chills, headache, fatigue, neck stiffness, myalgia, joint pain and swollen lymph nodes. There are likely hundreds of health conditions with significant overlap with these non-specific signs and symptoms. As spirochetes

disseminate from the site of the tick bite, additional EMs and manifestations can occur including 7th cranial nerve palsy, meningitis, or Lyme carditis [reviewed in (38)]. In the third stage, without proper treatment, patients may also experience neuroborreliosis or Lyme arthritis (LA).

The type and severity of LD manifestations are known to vary across infected individuals for reasons that are unclear but are likely attributable to both, differences in the infecting pathogen and the characteristics of the infected individual. They range from asymptomatic or subclinical infection (39–41) all the way to severe complications from LD that, in rare cases, result in death from Lyme carditis (42, 43).

EM Lesion

The characteristic EM lesion develops inconsistently across humans 3–30 days following a bite from an infected tick (44). The EM is often an annular, erythematous, expanding cutaneous lesion that may or may not have a central clearing. While it is sometimes referred to as a "bulls-eye rash," presentation is known to vary considerably (15, 45–47). Variation in skin pigmentation, as well as coloring and shape of the rash may also lead to missed or delayed clinical diagnoses (48). The central clearing in the rash is reported to be less common in endemic areas compared to non-endemic areas (15). While reports vary across studies, up to 30% of individuals diagnosed with LD do not develop an EM lesion (47, 49–52) or its presence is missed. If an EM lesion is absent, there is no clinically recommended laboratory test available to aid in the diagnosis of early LD because the currently recommended serologic tests are highly insensitive in the first few weeks of infection (51).

Direct Detection of Bb

Many bacterial infections are diagnosed using a variety of culture methods and the confirmation of pathogen identity through molecular techniques or differential biochemical assays (53, 54). This is not currently practical or feasible for LD. The direct detection of the pathogen in blood can be a challenge because of the narrow window of spirochetemia that is more likely during early infection and the low numbers of circulating *Bb* (55, 56). While the pathogen may disseminate from the site of the tick bite through the blood, it also disseminates through the lymphatics and is known to invade other more privileged tissues, such as the heart, nervous system, and connective tissue. *Bb* is a fastidious, slow-growing bacteria that requires up to 12 weeks of incubation in culture before a negative result is determined, which is too long to be useful in clinical diagnosis (32). In one study on the ability to detect spirochetemia in patients with EM through culture methods, they estimated 1 cultivable spirochete per 10 mL of whole blood (55). *Bb* culture also requires specialized skills and tools that most laboratories are not equipped with outside of the research setting, where these techniques remain valuable for basic science research (21). Finally, antibiotic treatment decreases culture positivity rates, making it useful only in untreated patients (21). Blood, serum or plasma is not a reliable tissue to detect *Bb* by PCR because the spirochetes are transient and in low copy number. Skin biopsy from the EM lesion is a more useful tissue diagnostically, but this step is invasive and patients that present with an EM lesion do not require laboratory confirmation for diagnosis of LD (21).

Emerging Diagnostics

For the reasons outlined above, there is an urgent need for pathogen-detection methods that are highly sensitive and specific and capable of reliably detecting infection by multiple pathogenic species of *Borreliella* and strains of *Bb* (see section Genomic Insights From Borreliaceae Lineages) at all stages of infection and disease (57). Of special concern are individuals with acute infection that do not present with an EM rash and have yet to generate a humoral response to *Bb*. The factors that control the development of EM rash also need to be delineated, along with a surrogate set of biomarkers to aid diagnosis of more complex cases of suspected LD. *Ixodes* ticks can carry multiple human pathogens (see section Transmission of *Bb* via Ixodes spp. Vectors), and diagnostic methods capable of detecting the most prevalent tick-borne pathogens and clinically relevant strains of *Borreliella* are needed. Promising new diagnostic methods are being developed using serology, direct detection assays, and other tests that measure host response to the pathogen. Select assays are outlined in **Table 2**. There is also a significant unmet need for diagnosing PTLD. Currently, this diagnosis is performed, in part, based on self-reporting of symptoms. Several groups are investigating correlation between blood transcriptome; blood metabolome; and gut microbiome of PTLD patients in search of potential diagnostic and causal markers. Of note are recent studies describing a distinct microbiome signature (58) and a blood metabolome signature (59).

Treatment

Recommended Treatment

Antimicrobial therapy for LD is often successful, especially when patients are treated in the early phase following detection of an EM lesion (79). As disease progresses, treatment must be extended and may be less effective (80, 81). Administration of doxycycline or amoxicillin for 14–21 days is the recommended treatment for early or early disseminated phase patients who do not have neurological involvement (44). Lyme arthritis is an indication of disseminated disease and the recommended treatment for this is the aforementioned oral antibiotic for 28 days. For patients with clinically evident neurological involvement, treatment with intravenous ceftriaxone is recommended. These suggested regimens are based on objective measurements. However, in the guidelines from the Infectious Disease Society of America (IDSA), the authors point out that "Response to treatment is usually slow and may be incomplete" (82).

There is widespread agreement in the medical community about the appropriate treatment of acute LD (83), however the appropriate treatment of patients meeting the Post-treatment Lyme disease syndrome (PTLDS) case definition remains a challenge due to incomplete knowledge about the condition and related uncertainties. Moreover, the recently updated IDSA guidelines for the treatment of LD remove mention of PTLDS altogether (83).

TABLE 2 | Emerging diagnostic assays for LD.

Test	Description	References
Serological assays		
TBD-Serochip	Array assay that discriminates antibody response to 8 tick-borne pathogens	(60)
mChip-Ld	Multiplex microfluidics assay targeting 3 Bb antigens for POC use	(61)
xVFA	Multiplex paper based lateral flow assay targeting 7 Bb antigens for POC use	(62)
Direct detection assays		
Karius test	Microbial cell-free DNA unbiased metagenomic sequencing	(63, 64)
PCR-ESI/MS	Direct detection PCR with electrospray ionization mass spectrometry	(24, 65)
Nanotrap urine	Nanotrap assay to measure *Bb* OspA c-terminus peptide	(66)
Host-focused assays		
Transcriptome	Transcriptome profiling by RNA-Seq to identify gene expression signature	(67, 68)
Proteome	Targeted mass spectrometry-based approach to identify protein biomarkers	(69)
SERA	Antibody repertoire analysis to identify Lyme specific motifs	(70)
ImmunoSEQ	T-cell receptor sequencing to identify Lyme specific signature	(71) [2]
Microbiome	Microbiome sequencing to identify Lyme specific signature	(58)
Metabolomics	LC-MS/LC-MS-SRM to analyze small molecule metabolites to develop biosignature	(59, 72–75)
QuantiFERON-Lyme	Assay that measures IFN-γ release from T-cells in response to *Bb*	(76)
Imaging		
Image Analysis	Deep learning algorithms that discriminate between EM and other skin lesions	(9, 77)
HS-198	A small-molecule fluorescent conjugate that targets a conserved protein in Bb for *in vivo* imaging.	(78)

Bb, B. burgdorferi; POC, Point of care; LC-MS, Liquid chromatography-mass spectrometry.

More clinical trials are needed to assess the efficacy and safety of drug regimens and complementary therapies for LD and its sequelae. This year, the first clinical trial coordinating center was established at Columbia University to facilitate the conduct of high-quality multi-site clinical trials and pilot studies related to LD and other TBDs.[1] Furthermore, tools capable of discriminating the etiology of persistent health issues following treatment for LD are needed in order to improve therapy development efforts and target treatments for more precise patient-centered care.

Drug Discovery and Preclinical Studies

Although early-stage LD can be successfully treated with doxycycline or amoxicillin, late-stage LD with arthritis and neurological symptoms can be refractory to antibiotic treatment. Wu et al. showed that stationary phase *Bb* are unexpectedly susceptible to cell wall synthesis inhibitors, and vancomycin in particular almost completely eradicates persisters *in vitro* (84) (see section Persistent Infection). Feng et al. recently identified FDA-approved drug candidates that are more effective at killing dormant *Bb* persisters *in vitro* than the current Lyme antibiotics (85).They found the drug combination Daptomycin + Cefoperazone (or Cefuroxime) + Doxycycline was most effective at eradicating *Bb* grown *in vitro* and more recently in the mouse model for *Bb* persistence (86, 87).

Complementary Therapies

Zhang et al. recently identified natural products and botanical medicines (*Cryptolepis sanguinolenta* (Yellow dye root), *Juglans nigra* (Black walnut), *Polygonum cuspidatum* (Japanese knotweed), *Uncaria tomentosa* (Cat's claw), *Artemisia annua* (Sweet wormwood), *Cistus incanus*, and *Scutellaria baicalensis* (Chinese skullcap) with good activity against both stationary phase and growing *Bb* (88). More recently, botanical medicines *Cryptolepis sanguinolenta, Artemisia annua, Scutellaria baicalensis, Polygonum cuspidatum*, and *Alchornea cordifolia* have also been shown to have activity against *Babesia duncani* (89). Since the above herbal medicines that have activity against *Bb* and *Babesia* have been used traditionally in patients with good safety profile, proper clinical trials are needed to evaluate their utility in treating patients with LD and coinfections. In addition, some essential oils such as oregano, cinnamon bark, clove bud, citronella, garlic, allspice, myrrh, hydacheim, and Litsea cubeba were shown to have excellent activity against both stationary phase and growing *Bb* (88, 90). However, further studies are needed to evaluate the active compounds in the essential oils, in order to elucidate the specific activity, to assess their potential toxicity and pharmacokinetic properties for activity against *Bb* infection in animal models before human use.

Well-Defined LD Patient Subgroups With Posttreatment Sequelae

Antimicrobial therapy effectively treats LD in most people, however some patients experience ongoing health issues following treatment (4, 91). Antibiotic-refractory LA is the most well-characterized subgroup of patients with posttreatment sequelae. In recent years, characterization of patients with PTLD, a well-defined subset of patients with persistent or chronic LD, has also advanced considerably. Next, we review progress in the description of each of these post-treatment sequelae.

[1] https://www.columbia-lyme.org/clinical-trials-network

Antibiotic Refractory LA

The LD patient subgroup with LA has been meticulously studied for decades. In 1975, a cluster of cases was brought to the attention of the Connecticut State Department of Health independently by two mothers concerned about the number of children with swollen knees with similarities to juvenile arthritis, a rare condition. An investigation revealed 51 similar cases (39 of these children) in Old Lyme, Connecticut and adjacent townships (92). By 1983, the causative agent and vector of LD were identified (93). LA—the proliferative and persistent synovitis of one or more large joints—is the most prevalent symptom in late-stage LD patients [reviewed in (94)]. LA may wax and wane months to years following untreated infection (95). Around 10% of LA patients experience persistent LA that does not resolve within a couple months after one or more rounds of antibiotic therapy (95–97). LA patients refractory to oral antibiotics are treated with intravenous ceftriaxone antibiotic therapy, with mixed results in adults (97, 98) and children (99). Persistent LA despite aggressive treatment with antibiotics is referred to as antibiotic refractory LA (100), or if no evidence of ongoing infection, post-infectious LA (101). Among patients with persistent Lyme arthritis, determining whether persistent joint inflammation is due to an ongoing antibiotic-refractory infection or a post-infectious immune response also raises important treatment dilemmas that are not yet resolved, as reviewed elsewhere (95). The true cause of antibiotic-refractory LA has remained enigmatic. Recent developments, however, suggest a complex interplay between both bacterial and host factors (102).

What bacterial components may contribute to inflammation after therapy? While the debate surrounding persistent infection continues (see section Persistence), undetectable levels of bacteria in the SF of some patients with antibiotic refractory LA has led to a search for alternative explanations for persistent inflammation. Intravital microscopy studies of antibiotic treated mice observed the persistence of *Bb*-derived material (103), which precipitated the possibility that bacterial debris may be responsible for immune activation. Later, we highlight a recent finding that a component of the bacterial cell envelope may exacerbate and prolong the initial response to the LD agent (see section Persistent Antigenic Debris).

PTLD

PTLD is a narrow but defined subset of patients with persistent symptoms following treatment for LD [reviewed in (30, 44)]. Patients with PTLD suffer persistent or relapsing symptoms, such as severe fatigue, cognitive issues, sleep disturbance, and musculoskeletal pain that negatively impact their functional abilities at least 6 months following treatment for LD. The reductions in quality of life for patients with PTLD are comparable to patients suffering from congestive heart failure (104, 105). These health problems can last months or years following the initial treatment for LD. The incidence of PTLD is as high as 20% in some patient populations (13, 44, 91, 106–108). Prevalence of PTLD is currently difficult to ascertain, due in part to the variability in the case definition applied in the literature and variability in reported rates of treatment failures among patients with LD, so estimates range from 69,000 to more than 1 million cases in the USA (107, 109).

Descriptions of patients with persistent symptoms following antibiotic treatment for LD date back to studies in the 1980's (110, 111) and 1990's (79, 80, 112, 113). The term "post-treatment [chronic] Lyme disease syndrome" and a case definition PTLDS were first described in 2003 (114) and 2006 (44), respectively. Today this case definition, along with standardized measurement of subjective symptoms (115), informs the eligibility and exclusion criteria for many studies and trials related to the broader and more heterogeneous population with chronic or persistent Lyme (91, 116, 117), so that outcomes from research studies may be compared and potential mechanisms of pathogenesis defined with less ambiguity (44). Recent studies have deployed deep phenotyping methods and identified multiple biomarkers that distinguish these patients from LD patients that return to health and/or controls, as described below. These studies provide evidence that PTLD is an infection-associated condition with a distinct biology. Here and throughout, we adopt the terminology PTLD, as previously proposed (44, 118), to describe this condition.

The diagnosis and treatment of patients with PTLD face significant challenges. Objective tests to diagnose PTLD currently do not exist. Instead, diagnosis is made clinically through a process of documenting a prior history of LD and excluding other potential causes of persistent symptoms. Currently there are no prognostic indicators to stratify LD patients for their susceptibility to PTLD, but elevated levels of the T-cell chemokine CCL19, IL-23 or muted B-cell response were recently reported to be more common among PTLD patients compared to those that return to health following initial treatment (119–121). For patients that progress to PTLD, there are no FDA approved curative therapies. Safety and efficacy data for off-label treatment regimens and complementary therapies for PTLD are not well-established. Objective tests to determine whether a patient is cured or responsive to therapy also do not exist.

Objective laboratory tests that improve the diagnosis and treatment of this patient subgroup appear to be on the horizon in part due to investments in the creation of carefully constructed cohorts and biorepositories of well-defined patients with PTLD (see section Biorepositories and Research Cohorts). For example, the first biomarker of PTLD to be identified in one cohort that was then confirmed in a second, independent cohort of PTLD patients in the USA was published in 2020. This metabolomic signature discriminates clinically cured LD patients without PTLD from LD patients with PTLD (59). Alteration of gene expression was shown to persist after treatment for early LD among a small sample of patients followed longitudinally for 6 months. While no transcriptome signature was identified that could discriminate between patients that return to health after treatment compared to those that do not, the signature may lead to new objective tests for early LD (67). A gut microbiome signature was also recently identified that could distinguish PTLD patients from both healthy controls and ICU patients, further suggesting that PTLD is a distinct and definable disorder even if the underlying etiology remains uncertain (58). Other biomarkers related to LD and PTLD are reviewed

below (section Pathogenesis). Taken together, these findings indicate that novel diagnostics and treatments for PTLD are now within reach.

FUNDAMENTAL KNOWLEDGE

Genomic Insights From Borreliaceae Lineages

Between 1982 and 2010, the *B. burgdorferi* species complex, known as *B. burgdorferi sensu lato*, steadily expanded from 1 to 18 species (sometimes referred to as genospecies) as isolates from tick vectors, their hosts, and patient samples were characterized [reviewed in (122)]. A subset of these species are associated with human disease. *B. burgdorferi sensu stricto (Bb)* in the USA, as well as *B. afzelii* and *B. garinii* in Eurasia are the most common agents of LD in the Northern hemisphere. Cases of LD in Europe are also caused by *Bb and B. bavariensis* (123), but are less common. *B. spielmanii* (124), *B. bisettiae* (125–127), and *B. lusitaniae* (128, 129) have been identified in human specimens but their clinical importance is less clear. *B. valaisiana* has been identified in human specimens (130), but others have recently provided compelling reasons why existing evidence does not support it being considered a human pathogen (131). Additional species have been identified in tick vectors or their hosts, but not in patient samples.

The genus *Borrelia* was recently divided into two genera (132, 133). This division groups the tick- and louse-borne relapsing fever (RF) agents and their relatives into the genus *Borrelia* and the LD agents and their relatives into the genus *Borreliella*. Historically, *Borrelia* species were classified according to whether they were transmitted by hard-bodied or soft-bodied ticks (134). The advent of molecular characterization through genetic analyses, and more recently, whole genome sequencing, has led to a refinement in our ability to classify spirochetes that are morphologically very similar. The division of the genus is based on phylogenetic and comparative genomic analyses using 38 public genomes of 18 different species. Due to naming standards, RF agents retain the genus *Borrelia* since they were the first species identified in the genus. The reclassification of *Borrelia burgdorferi sensu lato* species to the genus *Borreliella* means that their names will change but the abbreviations are retained, e.g., *B. burgdorferi* for *Borreliella burgdorferi (Bb)*. The proposal generated considerable debate across the scientific community (135, 136). While adoption of the new naming convention has thus far been low in recently published literature, canonical bacterial reference manuals (133) and scientific databases have begun to incorporate the change.

New clinically relevant *Borreliella* species continue to be discovered [reviewed in (134, 137), reviewed in (138), reviewed in (139, 140)]. In 2016, a new pathogenic species, *B. mayonii*, was identified in the upper Midwest of the USA through PCR analysis of more than 100,000 human tissue specimens collected between 2003 and 2014. Specimens from a total of six patients were deemed positive for *B. mayonii,* all presenting after 2012 with signs and symptoms consistent with LD (141). The genomes of two *B. mayonii* isolates have been sequenced, with notable differences from *Bb* (strain B31) including the absence of the complement inhibitor Cszp and dozens of other proteins (142).

Bb has one of the most complex genomes of any bacteria characterized to-date [reviewed in (143)]. *Bb* contains a single linear chromosome of ∼900 k base pairs (bp) with between 7 and 21 different plasmids, ranging in size from 5 to 84 kbp as reviewed from the genome sequences for 27 *Bb* isolates determined since the elucidation of the *Bb* B31 genome sequence in 1997 (144). The single chromosome appears to be very constant in gene content and organization across these *Bb* isolates. Overall, dozens of *Borreliella* isolates have been sequenced (144–147). These include the partial genomes of 64 isolates recently identified from collections across Canada, an emerging area for LD (148). To-date, chromosome assemblies have been reported from these isolates without, however, an equivalent analysis of the plasmid content. In the *Bb* genome, the conserved linear chromosome encodes most housekeeping genes, while the variable set of linear and circular plasmids encodes most of the outer membrane lipoproteins (149–151). Three plasmids, cp26, lp17, and lp54, are conserved across sequenced isolates of *Bb* to-date [reviewed in (143)]. The content and number of the plasmids however appear much more variable with some loss of these once *Bb* is *in vitro* cultured for an extended period of time. It is noteworthy that while extra-chromosomal DNAs are often referred to as plasmids—non-essential DNA that carries pathogenic and/or material that infer a selective advantage in a particular situation—some *Bb* "plasmids" carry essential genes and are more akin to mini-chromosomes. Knowledge about plasmids is currently limited due in part to the constraints next-generation sequencing technologies impose with short reads and the subsequent challenges with assembly. Long-read sequencing technology is poised to enhance our understanding of plasmid content and their dynamics across species and strains (150, 152).

Characterization of *Borreliella* isolates at the genome level is important to determine how genomic variation correlates with different disease phenotypes [reviewed in (122, 157)]. Molecular typing is used to classify distinct lineages of *Borreliella*. Common methods include pan-genome snp analysis (153), multi-locus sequence typing (MLST) (154), ribosomal RNA intergenic spacer typing (RST), and outer surface protein C (OspC) typing [(150), reviewed in (155)]. These methods for the classification of strains of *Bb* yield different numbers of distinct groups. Isolates of *Bb* from clinical samples collected from patients in the USA and Slovenia were recently compared and distinct differences were observed across genotypes, clinical manifestations, and inflammatory potential (156). In another recent study, serial blood samples obtained over a 21 day period from four patients with acute LD were assessed for infection with *Bb* via a novel direct detection method that combines PCR and electrospray ionization that is also able to discriminate different *Bb* genotypes. Two of 4 patients were infected with more than one genotype of *Bb*. Notably, the dominant *Bb* genotype changed over time in these two patients during antibiotic treatment (65). Infection with heterologous strains of *Bb* in humans is consistent with studies of pathogen burden in both ticks and vertebrate hosts (see section Transmission of *Bb* via Ixodes spp. Vectors). New technologies and high-throughput screening methods are

advancing our understanding of which genes in the *Bb* genome are critical for ecologically or clinically relevant phenotypes, such as infectivity, tissue tropism (157) or drug tolerance. In a series of recent experiments, transposon insertion sequencing (Tn-seq) was used to identify genes associated with mammalian infection, resistance to oxidative stress, and survival in tick vectors (158–160).

LD agents are believed to inflict damage to people through inflammation caused by their immune response. No exotoxins have been identified in the genome with similarity to any previously described exotoxin found in other bacterial species, and the necessary secretory components appear to be absent. In addition, unlike most classical diderms, *Bb* does not produce the endotoxin Lipopolysaccharides (LPS) (144). A genome-wide proteome screen for immunogens in *Bb* (B31) using sera from patients with natural infections found that about 15% of the 1,292 open reading frames evaluated code for products that are immunogenic (161). More than 120 lipoproteins are encoded in the *Bb* genome (162) and represent nearly 8% of open reading frames (163). Of 125 lipoproteins examined experimentally, 86 of these are secreted to the bacterial surface (162).

Proteomic Insights From Borreliaceae Lineages

The application of mass spectrometry-based proteomics has recently gained interest in the field of *Bb* and LD research to advance the understanding of disease pathogenesis and develop potential diagnostic methods and vaccines. Progress in *Bb* proteomics has been limited primarily to the proteins contained in the chromosome, while proteins encoded in the extra-chromosomal plasmids remain poorly characterized. In either case, attention has also been focused substantially on proteins encoded by genomic sequences of *Bb* that are homologs of B31—the vastly used isolate that was first collected from Shelter Island, New York (164). Further, very few laboratories working on proteomic workflows generate high-quality genomic data from different isolates of *Bb*. Nevertheless, advanced mass spectrometry-based proteomics provide unmatched information such as protein identification, quantification and post-translational modifications. The characterization of the *Bb* proteome enables basic science research and the development of vaccines and diagnostics (44, 165). Though several proteomic studies have been published, no high-resolution mass spectrometry (MS) data is represented in a publicly accessible repository for the community to access and utilize in research goals (162, 166, 167). The recent construction of the *Borreliella* PeptideAtlas repository (http://www.peptideatlas.org/) provides a unique community resource that contains large-scale assembly of observed and MS-derived validated data uniformly processed through the Trans-Proteomic Pipeline (TPP) (168). This database contains the proteome information from *Bb* isolates B31, 5A4, 297 and MM1 where 39,145 distinct peptides are validated and represent 1,283 *Bb* proteins. In addition to the unique peptide identification data, post-translational modifications are also presented with their validated MS spectra. The *Borreliella* PeptideAtlas is a dynamic proteome resource in terms of size and complexity and is continually updated as new datasets of *Bb* proteomes become available. These new datasets are ingested, processed through the TPP data analysis pipeline to ensure low false-discovery rates and presented in the conglomerated relational database for all users to explore and utilize. For example, the data can be mined to provide leading candidates across the isolates represented and the experimental conditions they were subjected to allowing exploration of how *Bb* adapts to, and is able to survive in a wide variety of environmental conditions. This resource provides a foundation for researchers to understand the dynamics of proteome organization throughout stages of infectivity and to generate targets to arrest infectivity.

Transmission of *Bb via* Ixodes Spp. Vectors

The black-legged ticks, *Ixodes scapularis* and *Ixodes pacificus* on the West Coast, are the primary vectors of *Bb* in the USA. In endemic areas, the proportion of *Ixodes spp.* ticks infected with *Bb* can be remarkably high. One recent survey of the pathogen burden of 197 *Ixodes scapularis* ticks collected from New York and Connecticut where LD is endemic, revealed 111 (56%) ticks were infected with *Bb* and 37 (19%) were co-infected with more than one human pathogen (169). The high pathogen burden is consistent with previous tick surveys in the same region (170). In contrast, the percent of infected ticks in other regions of the USA is much lower. In recently published surveys across California, for example, fewer than 5% of *Ixodes* spp. ticks were infected (17, 170). *Ixodes* ticks sometimes carry multiple strains of *Bb* (65, 150, 171–173) that may impact the course of disease in people that are co-infected. One tick survey showed that 39% of *Ixodes* ticks in North America are infected with multiple genotypes of *Bb* (170).

Ixodes scapularis ticks feed only once per life stage (larvae, nymph, adult) after hatching from eggs. They primarily acquire *Bb* through feeding on infected vertebrates that are mostly mammals, but also some species of birds (174). *Bb* spirochetes colonize and persist in the tick until some are transmitted to a new host during the next feeding, while others remain in the tick during molting and the next life stage (175). *Bb* spirochetes are not transmitted from the adult tick to the egg, or it may occur only rarely. While transovarial transmission (TOT) of *Bb* in *Ixodes spp.* ticks has been reported in the literature, these reports may be attributable to confusion between *B. miyamotoi*, a relapsing fever agent, and *Bb* (176, 177).

Bb spirochetes have evolved multiple mechanisms to aid survival in adverse environments throughout the enzootic cycle. While colonizing the tick gut they must impede immune defenses of the tick, those of the host blood meal, and coexist with other residents of the tick gut microbiome. After their migration from the tick gut through the tick body cavity (hemocoel) and into the tick salivary glands, *Bb* spirochetes are expelled during feeding into the next host where they must again impede immune defenses and change their gene expression to survive and establish infection in a vertebrate host. The diverse and dynamic interactions between *Bb* and *Ixodes* ticks during *Bb* colonization, persistence, and transmission was recently reviewed elsewhere (178), but several recent findings are worth highlighting here.

The stability, abundance and diversity of the tick gut microbiome in *Ixodes* spp. is unsettled (179, 180), but there is evidence that the microbiome impacts the ability of *Bb* to effectively colonize the tick gut (181). The *Bb* genome lacks interbacterial defense pathways, which suggests they may not thrive in polymicrobial environments and the presence of certain taxa may interfere with colonization and persistence in the tick gut. For example, *Pseudomonas* possess genes for a Type VI secretion system that can deliver toxins to bacterial and eukaryotic members also residing in the tick gut microbiome (179, 182). Consistent with these findings, ticks infected with *Pseudomonas* are associated with lower burden of *Bb* (179, 182). Future research on the tick microbiome may lead to insights about approaches to disrupt pathogen colonization and transmission.

The tick salivary glands are important for transmission of *Bb* spirochetes. Tick saliva is essential to adequate tick feeding and contributes to human infection [reviewed in (183), reviewed in (184), reviewed in (185)]. During feeding, a tick alternates between secreting saliva and ingesting blood meal. Tick saliva not only carries pathogens from the tick into the host, but also contains molecules that function to aid the feeding process by creating blood flow through vasodilation and anticoagulative properties (186), blocking pain and itch (187, 188), impeding wound healing (189), and suppressing host immune response (190, 191). The composition of proteins expressed in tick saliva changes during feeding, which suggests another potential mechanism for host immune evasion through antigenic variation (192). A greater understanding of the components of tick saliva that support the infectivity of *Bb* may provide new routes to the prevention of LD.

Pathogenesis

The pathogenesis of LD is believed to be driven in large part by the immune response of patients, although the underlying causes of ongoing inflammation and tissue damage in different stages of LD remain an active area of research. The microbial origin and inflammatory nature of untreated LD is better understood than the pathophysiology that underlies persistent signs and symptoms of disease experienced by some individuals following antimicrobial treatment. The dominant hypotheses about potential mechanisms underlying PTLD are immune inflammation and dysregulation and persistent infection and/or persistent antigenic debris. In this section, we review progress in the past 5 years on our understanding of pathophysiology of LD, starting with untreated LD. Then, we will review new evidence that has emerged related to persistent disease following treatment.

Immune Activation and Inflammation in Untreated LD

Inflammation is an important mechanism the body uses to aid in the elimination of pathogens. However, too much inflammation, such as what happens during a "cytokine storm" among some severe COVID-19 patients when cytokines are overproduced, can overwhelm the body and cause grave tissue injury. An aberrant inflammatory response is also what underlies autoimmune disease (193). In this section we review immune activation and inflammation associated with LD that begins soon after an infection with *Bb* from a tick bite.

The skin is an important first barrier to *Bb* infection. Interactions between the pathogen and human skin begin at the site of the tick bite where *Bb* spirochetes invade then disseminate outwardly, sometimes causing circular or elliptical EM lesions in their wake. One study induced suction blisters over EM lesions of LD patients in order to characterize the dermal leukocytes and cytokines of the aspirates from the skin. They found the aspirates to be enriched for T cells, monocytes/macrophages, and dendritic cells (DCs) compared to uninfected controls. Two cytokines, IL-6 and INF-γ, were predominant in the EM lesions (194).

The response of the innate immune system, including complement pathways and acute-phase proteins (APPs), occurs more or less immediately while the adaptive B- and T-cell response may take days to weeks. Macrophages and dendritic cells resident in the skin express a range of pattern recognition receptors (PRRs) that initiate signaling cascades and inflammatory responses as an early line of defense when they encounter and internalize a *Bb* spirochete [reviewed in (195)]. A yeast display screen of >1,000 extracellular and secreted human proteins identified direct interactions between *Bb* isolates and one human host factor, Peptidoglycan Recognition Protein 1 (PGLYRP1). *In vitro* assays show recombinant PGLYRP1 binds with purified peptidoglycan from *Bb* and have borrelicidal activity (196). In murine models deficient in PGLYRP1 ($PGLYRP1^{-/-}$), pathogen burden of experimentally infected mice were higher than wild-type in hearts and joints but not skin. Moreover, $PGLYRP1^{-/-}$ mice had reduced IgG serum levels and elevated proinflammatory cytokines (IFN-γ, CXCL9, CXCL10). The relationship between PGLYRP1 and pathophysiology of LD in humans is not yet known.

Phagocytic cells will attach to a spirochete via a repertoire of host cell surface receptors. These include Complement Receptor (CR) 3 and CD14 (197–200), urokinase receptors (uPAR) (200, 201), scavenger receptors such as macrophage receptor with collagenous structure (MARCO) (200, 202), some Toll-like receptors (TLRs), C-lectins, sialic acid-binding immunoglobulin-like receptors (siglecs), Fc receptors, or others (200). The attachment of *Bb* to host cell surface receptors leads to signaling that induces innate and specific adaptive immune responses, as well as clearance of the pathogen through phagocytosis. For example, the host cell surface receptor, Toll-like receptor 2 (TLR2), is a PRR that binds to ligands on the *Bb* cell surface that display certain pathogen associated molecular patterns (PAMPs). When a PRR senses a pathogenic ligand, it upregulates an inflammatory response, which commonly includes the induction of pro-inflammatory cytokines, chemokines, and antimicrobial peptides (AMPs); however, different types of activated PRRs induce different gene expression patterns. The production of pro-inflammatory cytokines, such as interleukin-6 (IL-6), recruits blood cells to the site of inflammation and induces the production of APPs, such as C-reactive protein (CRP).

The internalization and degradation of engulfed *Bb* spirochetes is sensed by intracellular receptors, including the NOD-like receptors (NLRs) and endosomal TLRs. These receptors activate signaling pathways that induce the production

of cytokines, including interferons (IFNs). These are classified into three main types: I (IFN-α or IFN-β), II (IFN-γ), and III (IFN-λ) [(203), reviewed in (204), reviewed in (205)]. Type I IFNs are induced by *Bb* DNA or RNA through TLR7 and TLR9 (203), as well as TLR8 in monocytes (206). Type II IFN is produced by innate lymphocytes, including natural killer cells (NK cells) and by T helper cells (Th) (207). *In vitro*, type III IFN is induced in peripheral blood mononuclear cells (PBMCs) through TLR7 by live spirochetes or purified *Bb* RNA (208), perhaps controlled by *Bb* plasmid lp36 (203). The role of the type III IFN pathway in disease pathogenesis has not been fully elucidated, although clinical isolates associated with disseminated disease produce stronger IFN responses (Type I and III) (203).

The early stages of LD show an elevation of inflammatory markers and immune mediators (69, 119, 209, 210). CRP is an APP that is used clinically as a marker of inflammation that may denote infection, malignancy or cell stress (211). Within a few hours of an infection or other stressor, cytokines secreted by immune cells will enter the bloodstream and cause the liver to secrete CRP. Generally, the normal range of CRP for healthy adults is <10 mg/L; moderate elevation is 10–100 mg/L; and marked elevation is >100 mg/L (212). A longitudinal assessment of CRP levels in serum samples from 44 LD patients presenting with one or more EM lesions and followed for 2 years after treatment, showed a significant elevation of CRP prior to treatment and a rapid decline to control levels following treatment (209). Elevated levels of CRP at the pre-treatment visit were seen again in a larger study by the same group, along with 6 additional elevated markers, including CCL19, ferritin, fibrinogen, IFN-γ-induced protein 10 (IP-10), monokine induced by IFN-γ (MIG), and serum amyloid A (SAA) (119). The first systematic study of APP levels in serum samples from patients at different stages of LD and healthy controls was recently conducted (210). Consistent with the previous studies, CRP levels were most elevated in patients with early localized (single EM, $n = 18$) and early disseminated (multiple EMs, $n = 17$) LD, with 33 and 71% of patients, respectively, having CRP levels >10 mg/L (210). More recently, a proteomic analysis of serum proteins in 70 LD patients with one or more EM lesions at the time of diagnosis, identified six proteins, including CRP, with significantly altered serum levels shared across two independent cohorts (69). The other five elevated proteins are APOA4, C9, CST6, PGLYRP2, and S100A9. Two independent studies have also shown that the chemokines, CXCL-9 and CXCL-10, both known to be associated with IFN-γ production and thus a "TH-1 type" of immune response, are elevated at time of diagnosis in EM+ LD patients in the USA and Europe (120, 209). Among a European cohort, patients with symptoms at study entry had significantly higher levels of CXCL9 compared to patients without symptoms (120).

Inflammation and Immune Dysregulation Among Patients With Persistent LD

The immune profiles of LD patients with persistent health problems following antibiotic treatment are not consistent across well-characterized subgroups, however several potentially important immune mediators have emerged within subgroups. Next, we will review each of these.

CRP in Patients With Persistent Symptoms Following Treatment

The levels of CRP in other stages of LD (subsequent to early localized or early disseminated, as described above) and patients with persistent signs or symptoms following treatment are less consistent. Among patients with antibiotic-refractory LA ($n = 11$), 55% have serum CRP levels >10 mg/L while patients with early neurologic, late neurologic, and antibiotic responsive LA showed no significant difference in CRP levels compared to controls. For patients with PTLD ($n = 74$), 15% had CRP levels >10 mg/L compared to 4% of patients with LD that returned to health following treatment ($n = 68$) (210). For some diseases, such as cardiovascular disease, serum CRP in the range of 3–10 mg/L is considered of clinical value for understanding an underlying inflammatory process or stratifying risk in some patients (213–215). A significant proportion of both, antibiotic-refractory Lyme arthritis (73%, $n = 11$) and PTLD (55%, $n = 74$) patients have serum CRP levels in this range (210). While these data are suggestive of an ongoing inflammatory process in these two patient subgroups, more research is needed to better understand the role of CRP, along with other markers of inflammation, and the underlying mechanisms at work in affected patients.

IFN-γ and Antibiotic-Refractory LA Patients

Antibiotic-refractory LA patients without evidence of an ongoing infection, show excessive IFN-γ production. So far, this biomarker appears to be specific for LA as serum levels of IFN-γ are not significantly elevated among EM+ LD patients at the time of presentation compared to controls, nor are levels of this cytokine significantly elevated in patients with PTLD (119). A recent study did a comparative analysis of synovial tissue from patients with LA, rheumatoid arthritis, and low inflammation osteoarthritis and found that LA is unique, showing elevated levels of IFN-γ and IFNγ-producing T cells and NK cells in synovial fluid (216). This study also identified and characterized a population of fibroblast-like synoviocytes (FLS) that are hypothesized to be involved in mediating persistent inflammation. These cells, when activated by IFN-γ *ex vivo,* expressed genes and pathways that overlapped with that seen in postinfectious LA synovial tissue (216). This suggests that the FLS are driving ongoing inflammation and suppressed wound healing in an IFNγ-dependent manner.

CCL19 Among PTLD Patients

The T-cell chemokine, CCL19, was associated with susceptibility to PTLD. In a recent study, sixty-four cytokines, chemokines, and inflammatory markers were measured in serum collected from 76 EM+ LD patients at six visits over 1 year. Eleven patients (14.5%) went on to develop persistent symptoms that impacted daily functioning following treatment and were classified as having PTLD. Twenty-nine patients (38.2%) had symptoms following treatment without a functional impact on daily living and 36 patients (47.37%) returned to health. Patients with CCL19 above

111.67 pg/ml at the (1-month) visit were 12.6 times more likely to meet criteria for PTLD at the 6 and 12 months timepoints (119). In a murine model, CCL19 along with IL-23, were associated with a pathological TH17 response in experimental autoimmune encephalomyelitis (EAE) (217). However, it has not been fully assessed to which extent the TH17 pathway is induced in LD patients.

IL-23 Among European PTLD Patients

A cytokine, IL-23, associated with IL-17 production and thus "TH-17"-type responses, is elevated in early LD and remains so among many European patients that have persistent symptoms following treatment. Eighty-six EM+, untreated LD patients that enrolled in an antimicrobial drug trial in Europe and were followed longitudinally for a year after treatment and were assayed for 26 cytokines and chemokines at 4 time points. Among the patients studied, 45 had symptoms following treatment consistent with having PTLD. One cytokine (IL-23) and two chemokines (CXCL9 and, to a lesser extent, CXCL10) showed significant differences across groups. Most patients with detectable IL-23 levels at study entry went on to develop PTLD and IL-23 levels remained elevated in these patients with ongoing symptoms. The seven patients with the highest levels of IL-23 ($\geq$230 ng/mL) all went on to develop PTLD. Thus, an aberrant TH17-related immune response might be contributing to symptoms in patients with elevated IL-23 (120). It is interesting to note that no significant difference was identified in serum levels of IL-23 among the longitudinal cohort of LD patients from the USA, while CCL19 was not noted to be significantly elevated in the European cohort (119, 120). One potential reason for these differences is the difference in the primary agents of LD in Europe and the USA: *B. afzelii* and *B. garinii* vs. *Bb*. However, the mechanisms underlying these and other differences between LD in North America and Europe remain poorly understood.

Autoantigens and Self-Reactivity in LA Patients

The origin of autoimmune disease (AD) in humans have previously been thought of as triggered by microbial infection, which may serve as a catalyst for development of responses to self-antigens [reviewed in (218)]. Several autoimmune diseases are associated with bacterial triggers, such as gastric autoimmunity and Guillain-Barré syndrome triggered by infection with *H. pylori* and *C. jejuni*, respectively [reviewed in (219)]. One proposed mechanism for possible LD-associated autoimmunity is the circumstance where sequence or structural homology between human and *Bb* proteins cause B- and T-cell receptors to cross-react with an epitope on a *Bb* protein (the intended target) and a human protein (the unintended target). This could lead to ongoing inflammation of tissue and has been most well-studied in patients with LA (220–228). Four self-proteins (autoantigens) recognized by CD4 T cells have recently been identified in LD patients with LA through the use of tandem mass spectrometry on peptides in complex with MHC class II receptors (HLA-DR) (229). The four autoantigens identified include peptides from endothelial cell growth factor (ECGF), apolipoprotein B-100 (apoB-100), matrix metalloproteinase-10 (MMP-10), and annexin A2 proteins. Autoreactivity to these self-proteins appear to be primarily associated with LD, except annexin A2, which was associated with other rheumatic diseases (228), and potentially, to severe COVID-19 (230). To-date, no specific autoantigens have been identified reliably in LD. For the autoantigens that have been identified, we currently do not know whether the observed autoreactivity is induced by the presence of specific *Bb* antigens.

Persistence

Persistent Antigenic Debris

Recent studies of *Bb* peptidoglycan have renewed interest in the potential roles this immunogenic macromolecule may play in LA and, more broadly, in disease pathogenesis. Peptidoglycan (PG) is an essential biopolymer that acts like a molecular bag—surrounding the bacterial cytoplasm and preventing cell bursting due to osmotic pressure. The cell walls of virtually all bacteria contain PG, and chemical and structural conservation is apparent [reviewed in (231)]. Glycan strands, made up of the repeating disaccharide *N*-acetyl-glucosamine and *N*-acetylmuramic acid, are cross-linked by peptides composed of often alternating L and D-amino acids. Deviations from this chemical and conformational arrangement are rare, which make PG a quintessential pathogen-associated molecular pattern (PAMP) [reviewed in (232, 233)]. Recognition of bacterial peptidoglycans by innate immune system receptors [e.g., Toll-like receptors (TLRs), PG recognition proteins (PGRPs), and NOD proteins] leads to inflammation and the production of cytokines that can result in host tissue damage. Immune response(s) to bacterial PGs have been associated with symptoms of infections such as gonorrhea (234, 235), chronic gastritis, and pertussis (236). There has been some evidence for a potential role for peptidoglycan in several autoimmune diseases, including rheumatoid arthritis (237) and multiple sclerosis (238, 239).

The cell envelope of *Bb* contains a peptidoglycan (PG^{Bb}) that so far appears to be chemically unique, but conserved amongst some spirochetes. For example, close *Borreliae* relatives in the Relapsing Fever clade and *Treponema* genus have been reported to possess L-Orn-type PG (240, 241). For bacteria to grow, divide, and ultimately cause disease, PG is continuously remodeled—small muropeptide fragments are removed and replaced with multimers. Unlike many other bacteria, *Bb* lacks the genetic components necessary for recycling the excised muropeptide fragments back into the cytoplasm. Instead, muropeptides are shed during growth and accumulate in logarithmic fashion that correlates with spirochete density (102). Analysis of radio-labeled, PG-associated amino acid over time, indicates that *Bb* sheds ∼45% of its PG per generation (102). Despite the overall abundance PG shedding would cause, its role in LD pathogenesis remains to be fully elucidated.

PG^{Bb} elicits an immune response in humans. This was first shown in 1990 when PG isolated from *Bb* was injected subcutaneously into the forearm of a volunteer, one of the co-authors of the report, who then experienced intense inflammation at the injection site for 72 h (242). Jutras et al. detected PG^{Bb} in 94% of synovial fluid samples collected from 34 patients with LA (102). They also demonstrated the connection between PG^{Bb} and disease pathogenesis through tail

vein injection of PG^{Bb} into mice and the subsequent observation of acute arthritis (102).

Much remains to be determined about the role of PG^{Bb} in LA and, more broadly, in LD. For instance, are the PG remnants that of dead/dying bacteria following phagocytosis or antibiotic therapy or are they shed muropeptides? Transcript levels of Lysozyme—the human enzyme responsible for degrading PG—are elevated in LA patient SF (216), so why isn't it working or is the substrate absent (polymeric PG vs. muropeptides). The fate of shed muropeptide fragments, or their dwell time in different anatomical sites, is not known. In fact, the exact chemical composition of released muropeptide(s) is yet to be determined. Since released muropeptides must contain L-Orn (102), one intriguing possibility is that PG chemistry affects the response and/or half-life in the human host. Do germline variants or differential expression of human PGLYRP1 impact the pathogenesis of LD? Clearly, much remains to be elucidated, but methods toward preventing the human responses to PG^{Bb} or eliminating the lingering antigen entirely, are two attractive avenues of future therapy in patients with persistent LD.

Persistent Infection

Like most bacteria, *Bb* are able to change their cellular phenotype in order to better survive in adverse conditions. *Bb* form persister cells, *in vitro* (85, 243) or when exposed to antibiotics [(243), bacterial "persistence' reviewed in (244), reviewed in (245, 246)]. The connection between persister cells, atypical morphological forms of *Bb* and disease pathogenesis in LD remains poorly understood [reviewed in (247)].

Antibiotic resistance vs. antibiotic tolerance. Generally speaking, the ability of bacteria to grow in the presence of an antibiotic indicates resistance of a specific nature. Whether the antibiotic targets bacterial protein or nucleic acid synthesis, cell wall synthesis or integrity, or a specific metabolic pathway, uninhibited growth in the presence of that antibiotic demonstrates that the bacteria have acquired the ability to counteract that drug. Resistance may be inherent in the genome, acquired by horizontal gene transfer, or produced by mutation. *Pseudomonas aeruginosa*, for example, possesses multiple operons that encode efflux pumps (248) and *Mycobacterium tuberculosis* has become resistant by virtue of mutations which, for example, prevent the inhibitors of cell wall synthesis from binding to their target enzyme (249, 250). Alternatively, bacteria may stunt their own replication in the presence of a bacteriostatic antibiotic, thus minimizing the effect. The latter is non-specific and can be referred to as antibiotic tolerance. *Bb* encodes an efflux pump system, but specific resistance to antibiotics has not been clearly demonstrated (251). The generation of slow-growing or non-growing "persister" cells *in vitro* is a well-established observation for multiple bacterial species (252, 253). *Bb,* in particular, has been shown to tolerate multiple antibiotics (85, 243) and form persisters by a stochastic mechanism leading to a slow-growing phenotype (254). Persister cells *in vivo* have not been demonstrated. In natural infection, the ability of bacteria to establish dormancy is perhaps best exemplified by *Mycobacterium tuberculosis*. Such infections can be latent for years and often never result in fulminant disease; the entry into dormancy is likely influenced by hypoxia or other environmental stressors (255). Another pathogenic spirochete, *Treponema pallidum,* can enter a chronic dormant phase within the human host and reactivate as tertiary syphilis years after the primary infection (256). How or where *T. pallidum* persists is not known.

If entry into a dormant phase occurs *in vivo*, the possibility for bacteria to tolerate growth-inhibiting compounds is a legitimate possibility. *B. burgdorferi* may enter dormancy during long periods of nutrient deprivation within the tick or following treatment of a mammalian host with a bacteriostatic antibiotic such as doxycycline. The stress response of the bacteria to nutrient deprivation has been described (257) and this capability to survive harsh environments may well contribute to antibiotic tolerance as well (258).

Bb infection, in particular. The question of the effectiveness of antibiotic treatment for LD has been contentious among physicians and researchers for some time (91, 259–261). Among the challenges to determining if antimicrobial therapy is curative is the absence of reliable measures to determine that infection has been cleared from LD patients and the vague, non-specific symptoms with which patients present in PTLD (30, 106). The notion that spirochetes may persist in humans derives primarily from the proportion of patients who experience symptoms post-treatment (107, 262). A few studies have examined this phenomenon in humans—two in the U. S. [reviewed in (263)] and one in the Netherlands (264). For both studies in the USA, only patients that had been treated for acute (early) LD were included and the authors concluded that resolution of symptoms did not occur with a subsequent 90-day treatment with antibiotics (265). While the Netherlands study also showed no significant difference between longer term treatment of patients with chronic symptoms and short-term therapy, the inclusion criteria were less stringent, potentially allowing patients without LD into the study. More recently, spirochetes were detected in post-mortem brain samples collected from a patient who previously was diagnosed and treated for LD and subsequently experienced chronic symptoms, including dementia (266).

Studies of persistence. To date, multiple studies in animals have shown that *Bb* spirochetes do persist following antibiotic treatment of a disseminated infection (267–269). Even in humans, rare evidence of possible persistence was gleaned from feeding uninfected ticks on a patient with PTLD (270). In this study, only *Bb* DNA was detected while attempts to visualize or culture viable spirochetes from the xenodiagnostic ticks were unsuccessful. As others have noted, without the recovery of metabolically active spirochetes, this experiment is suggestive but is not a clear demonstration of persistent infection in humans (271).

Evidence from experiments performed in mice and dogs reveals that spirochetes can persist in the mammalian host after the administration of antimicrobial drugs. In one study, dogs were treated with a 30-day course of amoxicillin or doxycycline 2 months after infection (267). Spirochetes were recovered from tissues in 3 of 12 dogs. Skin punch biopsy samples

from nearly all dogs were PCR-positive for *Bb* after treatment. Interestingly, serum antibodies to *Bb* declined post-treatment, but after the dogs were kept 6 months in pathogen-free housing, their antibody titers rose, indicating recrudescence. Spirochetal persistence has been examined in mice using xenodiagnostic studies in which naïve ticks were placed on infected mice that received a course of antibiotics (272–274). Those ticks acquired spirochetes from the mice post-treatment, detected by fluorescent imaging (272) or PCR (273, 274). Two of these studies examined the existence of persistent spirochetes as a function of the time elapsed prior to treatment (273, 274). Spirochetal DNA was more frequently detected in xenodiagnostic ticks (XT) that fed upon mice treated 4 months post-infection than from those treated 3 weeks post-infection. When XT that had acquired organisms from antibiotic-treated mice fed upon naïve mice, the mice harbored spirochetal DNA in multiple tissues (detected by PCR), but organisms could not be recovered by tissue culture. Finally, studies in primates have shown that morphologically intact spirochetes can persist following antibiotic treatment (268). In a subsequent study, not only were the spirochetes found intact after treatment, but were shown to be transcriptionally active and were detected in multiple tissues, including the brain and heart (275, 276). This is evidence to suggest that the organisms are not fully cleared and may be attenuated for infection or for recovery by tissue culture.

With rare exception (127), only *Bb* genetic material (DNA or RNA), antigen, or non-culturable spirochetes have been detected following antibiotic treatment of an established infection. In none of the aforementioned animal studies has the pathogen been recovered as indicated by spirochete replication in culture soon (1–2 weeks) after inoculation of the standard BSK medium with tissue or tick specimen. Some experts in the field have therefore surmised that the spirochetes are non-viable and therefore that the infection is not persistent (103, 271, 277, 278). Evidence of resurgence in mice that were evaluated a year after antibiotic treatment contradicts this notion of non-viability (269). In that study, the amount of *Bb* genetic material in each mouse was quantified and found to increase from a very low level a few months after treatment to levels as high as in untreated animals at 12 months after treatment, indicating that the spirochetes replicated. The *Bb* bacteria present after antibiotic treatment, which are metabolically active and appear to be capable of resurging *in vivo* or resuscitated under the right culture conditions have been deemed "viable, but non-culturable (VBNC)."

The VBNC state is not unique to *Bb*, but rather, it is known to occur in over 100 other bacterial species studied to date (279). Entering dormancy of this "nonculturable" type is very common for bacterial pathogens (280, 281). The VBNC state has been characterized as a deeper state of dormancy than that of persister cells, observed in several *Vibrio* species, *E. coli*, *Campylobacter*, *Burkholderia*, *Listeria*, *Salmonella*, and *Helicobacter* (281–283).

Persistent infection with *Bb* is difficult to rule-in or out as an explanation for LD patients with ongoing symptoms due to the challenge of culturing viable spirochetes from human specimens except in the earliest stages of infection, prior to antibiotic treatment (21). The failure to reliably isolate metabolically active spirochetes from patients does not exclude the possibility that they exist in some patients with ongoing health problems.

PREVENTION

Ecological Prevention

In the USA, *Bb* is the most common vector-borne pathogen; LD comprises 62.6% of all vector-borne diseases and 81.2% of all tick-borne diseases (1). There is an increasing trend of new cases in counties and states neighboring high-incidence regions, indicating a spread of the pathogen and disease risk in new geographical areas (52). The current complexities around the diagnosis and treatment of LD and PTLD suggest a growing need for primary prevention and to understand the intricacies of the ecological factors that impact disease risk.

LD risk, and the broader goal of prevention of LD, is commonly viewed through two lenses: an ecological approach that focuses on characteristics of the tick vector, its hosts and the pathogens it transmits; and a human behavior approach that examines how behaviors and attitudes of human individuals, such as frequency of outdoor activities or use of protective equipment, change risk to disease (284, 285). Indirect factors regarding host populations, abiotic conditions, and land use or land coverage have also been found to increase disease risk, yet the magnitude of impact or relation to tick encounters and infection risk is still not fully understood (284, 286).

Popular ecological preventative techniques gravitate around reduction of host populations, reduction of ticks, and reduction of pathogen infection in ticks or hosts. Popular human-behavioral strategies include altering the risk of exposure of humans through behavioral changes associated with self-protection, use of outdoor space, and modifications of the environment. White-footed mice are the primary reservoir for *Bb* and their density has been shown to affect LD risk (287–290). The culling of white-tailed deer is a common preventative technique, but research sheds doubt on the viability or practicality of mass culling, suggesting that the technique is only effective on islands or closed populations where complete elimination can be accomplished (291). Personal protective measures, including checking body for ticks and use of tick repellent are frequently promoted by government and public health agencies. Some of these measures have been shown to reduce disease risk, yet effectiveness may be as low as 20–40%, with some practices like checking one's body for ticks being found ineffective (284, 292, 293). One challenge to prevention is the fact that nymphal ticks are as small as a poppy seed, and their bites can easily go unnoticed. Land usage or cover has shown strong trends of being impactful on exposure to LD, but still being researched is the spatial scale of land usage (284). Questions of land use in residential spaces or neighborhoods are still being explored, as well as the human movement within those spaces (294).

Several research projects have been initiated to further explore LD prevention by minimizing infection risk. The Tick Project is undertaking an immense randomized control trial of effectiveness of *Met52* fungal spray and Tick Control System rodent bait boxes for LD prevention across 24 neighborhoods in Dutchess County, New York, while also collecting and assessing

data on the entomological and host population risk factors, tracking tick encounters, and documenting cases of LD and PTLD across these neighborhoods (295). New models are being created to predict the first incidences of LD in counties without any reported cases of LD based on abiotic and human behavior factors (296). Prediction models will need to account for climate change as a contributing factor to the expanding range of LD. In addition, there are still several gaps in our knowledge about effective preventative techniques that should be further studied: abiotic factors and the capability of predictive modeling, diversity of *Bb* strains in tick and host populations and its impact on disease risk, predator communities and their role on host communities, changing landscapes and urban spaces, and the costs, sustainability, and acceptability to the public of preventative techniques (286, 288, 290, 297).

Human Vaccine

There is an urgent need for a safe and effective human vaccine that targets multiple pathogenic *Borreliella* species and strains, or even more broadly, common co-infections. Currently, there is no human vaccine for LD commercially available in the USA. In 1998, the FDA licensed Lymerix, a recombinant Osp-A based vaccine for the prevention of LD in adults. The vaccine required three shots over two tick seasons and was reported to be 76% effective in the prevention of LD after the third shot (49). The mechanism of action of this and other Osp-A based vaccines is antibody-mediated blocking of the transmission of *Borreliella* spirochetes from infected ticks while feeding on a human host (298, 299). Public demand and acceptance of the vaccine was low for a variety of factors reviewed elsewhere (300–304) and Lymerix was pulled from the market by its manufacturer, Smith-Kline Beecham in 2002. Interest from industry waned and other efforts to develop Lyme vaccine candidates were also abandoned, including those by MedImmune, Baxter and Connaught Laboratories (300).

Twenty years after Lymerix, there are now multiple efforts underway to develop next-generation human vaccines for the prevention of LD. One challenge that these vaccines must address is the genetic diversity of pathogenic *Borreliella* species and strains across different geographies. Even a single infected tick may carry multiple heterologous strains of Bb. Therefore, current vaccine candidates incorporate multiple immunogenic antigens or multiple serotypes of a single immunogenic antigen. Outer surface proteins, especially OspA-C, are the most common antigens selected among current human vaccine candidates, but other antigens and vaccine strategies are being studied [reviewed in (305)].

VLA15 is currently the only Lyme vaccine candidate in human trials. VLA15 uses recombinant outer surface protein A (OspA) from six different OspA serotypes of pathogenic *Borreliella* species, including *Bb* (OspA serotype 1), *B. afzelii* (OspA serotype 2), *B. garinii* (OspA serotypes 3, 5, 6), and *B. bavariensis* (OspA serotype 4) (306–308). In a similar approach, a prototype vaccine that uses bacterial ferritin nanoparticles fused with seven serotypes of OspA molecules from *Bb*, *B. afzelii*, *B. bavariensis* and *B. garinii* recently showed durable high-titer antibody response in both mouse and rhesus macaque animal models (309). In both vaccine candidates, the antigenic residues in Osp-A serotype 1 that were previously suspected (221) but never shown to be cross-reactive were removed (304, 309).

There are several promising strategies for the development of other novel human vaccines for LD in experimental and preclinical stages. Marconi et al. has conducted studies in dogs on a subunit vaccine that includes OspA and at least 14 immunogenic linear epitopes ("chimeritope") from diverse isotypes of OspC. These outer surface proteins are expressed while *Bb* is in the tick gut (OspA) or during early human infection (OspC) [(305, 310–312), reviewed in (313)]. The OspC chimeritope is a component of the most widely used Lyme vaccine for dogs (314). By combining antigens that are expressed by *Borreliella* at different stages of infection, this vaccine has the potential to protect against spirochetes that are not blocked or killed while in the tick gut. This vaccine has not yet entered human trials. Another experimental vaccine targets Cspz, an outer surface protein involved in complement evasion (315).

The development of an anti-tick vaccine is one potential approach to protect people from multiple tick-borne diseases, including LD, as recently reviewed elsewhere (316). *Ixodes scapularis* ticks transmit 16 human pathogens (317) associated with tick-borne disease in the USA, including *Bb*, *Borrelia miyamotoi* (318, 319), *Babesia microti* (320, 321), *Anaplasma phagocytophilum* (320, 322), *Ehrlichia muris-like agent* (EMLA) (323) and Powassan virus (324, 325). During transmission to a human, bacteria interact with tick proteins in the gut and salivary glands. These interactions can influence whether transmission occurs. Increased protection might be conferred if any of several steps in the transmission cycle are inhibited by targeting one or several of these tick proteins simultaneously. For example, mice that were given antiserum to the tick protein, Salp15, and then were challenged with *Bb*, showed protection from colonization (326). Tick proteins may also elicit "tick immunity," a process during which a host becomes resistant to tick bites because the ticks cannot feed properly (327). If a vaccine can be developed that creates tick immunity in humans, this may enable the prevention of LD, and other tick-borne diseases, especially for those that migrate slowly from tick to human (305, 328–332). Viruses, such as Powassan, can be transmitted from tick to the human host in mere minutes. The attachment time required for tick-borne pathogens to migrate from tick to host was recently reviewed elsewhere (333). Encouragingly, there is already one commercially available tick vaccine used for the protection of livestock against tick infestation, though not including *Ixodes spp.* ticks (334–336).

FIELD BUILDING

To improve our ability to better address Lyme and other tick-borne diseases, we need to attract researchers to the field and build shared resources that accelerate research progress such as biorepositories, genomic resources, animal models, and preclinical services (57). Interest in LD research over the past 5 years has remained relatively flat: the term "Lyme disease" is mentioned in around ∼1,000 publications per year combined

from PubMed Central in the USA and Europe. In this section, we describe several key resources for investigators seeking to study LD.

Biorepositories and Research Cohorts

Well-characterized samples are an essential tool to help researchers develop and validate new diagnostic tests and to better understand the complexities of LD. Well-characterized sample sets can benefit medical providers, test developers, and the public at risk for LD (337). It is critical that sample users understand the criteria used to enroll participants, how samples were collected and stored, and what additional clinical and testing data may be available. Additional benefits can be realized when multiple sample users (test developers and researchers) are using the same well-characterized sample sets. Current sample sets available for researchers include the CDC Lyme Serum Repository (LSR) (337), the Lyme Disease Biobank (23), and samples from the Studies of Lyme Immunology and Clinical Events (SLICE) at Johns Hopkins University School of Medicine. Additionally, some investigators also have their own sample collections with, in some cases, blood samples, skin biopsy specimens and synovial fluid which form the basis for collaborative studies (69, 102, 338).

Lyme Disease Biobank

The Lyme Disease Biobank is a collection of more than 900 human biological samples that facilitates research in the field of LD and other tick-borne infections (TBI). Whole blood, serum and urine samples are collected from individuals presenting with the signs and symptoms of early LD with or without an EM lesion, individuals with later stages of LD including persistent LD, and unaffected individuals (endemic controls). Samples have been collected from the East Coast, Upper Midwest, and California. Robust clinical information accompanies the samples, including information about symptoms, EM (if present), current medications, history of LD and other TBI, medical history, and demographics. Photographs of EM lesions are also taken (if present). Participants enrolled with early LD also have the option of providing a convalescent sample 2–3 months after the initial blood draw. PCR testing is performed, in a blinded fashion, to confirm the presence of *Bb*, *Anaplasma phagocytophilum*, *Babesia microti*, and *B. miyamotoi* in the samples. Serologic assays for standard two-tiered testing analysis (ELISA followed by IgM/ IgG Western immunoblotting) have also been performed. Each participant's donation provides samples for ∼50 research projects, with aliquots of whole blood (1 and 2 ml), serum (250 μl), and urine (1 ml) available to sample users in academia and industry. More than 10,000 aliquots have been distributed across 50+ diagnostic research projects. The characterization of samples from 550 participants enrolled on the East Coast and Upper Midwest is detailed here (23). Specific panels are also available, including an unblinded panel for projects earlier in development, panels of later stages of LD including Lyme carditis and neuroborreliosis, panels of other TBI, as well as samples from patients with persistent LD. A tissue bank was recently launched for post-mortem and surgical samples. Tissue donors have the option to link their MyLymeData registry profile to their tissue sample (339, 340).

Lyme Disease Research Center

The primary focus of the Johns Hopkins Lyme Disease Research Center (LDRC) is clinical translational research to advance the fundamental understanding of LD through the characterization of carefully constructed cohorts of LD patients and controls, as well as a clinical biorepository of blood and tissue biospecimens. The LDRC enrolls participants from an expanded Mid-Atlantic region into a variety of research protocols, which all collect detailed health histories, clinical, and behavioral data. Over 350 participants have been enrolled in ongoing longitudinal cohort studies (some followed for up to 10 years), which include patients meeting CDC criteria for early and late LD, as well as uninfected controls without LD. An additional 275 participants meeting the IDSA definition for PTLD have also been enrolled in a clinical case series study (44). The SLICE studies obtain a number of different biosamples including: a skin biopsy (in patients with acute LD), whole blood, serum, plasma, PBMCs, DNA, RNA, skin and fecal swabs, and most recently, urine. All these samples are processed in the laboratory, aliquoted, archived and stored. To-date, they have shared ∼6,000 sample aliquots for collaborative initiatives. The center collaborates with key investigators who utilize these samples for immune profiling (209), transcriptomics (67, 68), proteomics (69), metabolomic (75), and microbiome-based studies (58). These studies generate rich and deep clinical and molecular data sets that may allow for new insights into LD pathophysiology, lead to new diagnostic and therapeutic approaches, and have contributed to the characterization of LD and PTLD longitudinally across dozens of clinical and neurobehavioral variables. The LDRC has demonstrated that PTLD is a definable condition that is distinguishable from those that "return to health" following infection and treatment for LD. With the ability to compare PTLD patients with controls uninfected with LD, the SLICE studies show that the rates of individuals with both symptoms and a decline in health-related quality of life are significantly higher in patients previously treated for acute LD than in controls (14 vs. 4%) (unpublished data). The growth of these cohorts over the years is also evidence that it is possible to not only recruit patients with PTLD into research, but also maintain their participation at high levels.

Long Island Outdoor Worker Cohort

To investigate the seasonal incidence and seroprevalence rates, a team of investigators at the Stony Brook School of Medicine assessed outdoor workers in the Hispanic/Latino immigrant population residing in Eastern Long Island and compared rates to those of non-immigrant outdoor workers. To further investigate occupational risk, they looked at differences of incidence rates in field workers and non-field workers within the Hispanic/Latino immigrant population. The study shows a significantly higher rate of *Bb* exposure among Hispanic/Latino immigrant field workers compared to those belonging to other occupations and in non-immigrant outdoor workers and also sheds light on the epidemiology, seroprevalence, and seasonal incidence of Lyme and other tick-borne diseases, as well as

their clinical manifestations. Treatment and prevention of LD in this population can be especially difficult to obtain when multiple barriers are in place, such as poor health literacy, lack of preventative measures and limited access to healthcare in those with more risk. These findings underscore the necessity of improved education and preventative measures to better protect this vulnerable population (341, 342).

Data Repositories

LymeMIND Commons

The LymeMIND center at the Icahn School of Medicine at Mount Sinai is developing LymeMIND Commons (https://commons.lymemind.org/), an online database and a search engine that contains collected data and metadata integrated from the consortium and other LD resources. LymeMIND Commons enables researchers with the ability to find and analyze various types of data and metadata related to LD. Beyond transcriptomics, data includes protein arrays, methylation profiling by high throughput sequencing, genotyping, and other platforms. The metadata in LymeMIND Commons is JSON serialized and hosted using the Signature Commons platform. The metadata within LymeMIND Commons is linked to external ontologies and other resources for each study. In summary, LymeMIND Commons serves as a unique resource to advance LD research.

DISCUSSION

More research into the prevention, diagnosis and treatment of LD is needed in order to address the significant health risks posed by this tick-borne disease. The annual NIH and CDC investment in Lyme and tick-borne diseases research has been relatively unchanged for decades and is small compared to many other infectious diseases. In 2015, the Steven & Alexandra Cohen Foundation established a research consortium involving over 30 leading universities, research laboratories and other organizations that aim to advance Lyme and tick-borne disease diagnosis and treatment, human vaccination, awareness and education, data science and management, and ecological prevention. Philanthropic funding, including a new public-private partnership around novel diagnostic technologies, is critical to address the historically small amount of federal funding for LD compared to some other infectious disease of public health concern. While the Kay Hagan Tick Act did recently boost federal support for LD research (343), more is needed.

LD is a large topic. Some important advances were excluded for space considerations, such as immune evasion by *Bb* spirochetes, common co-infections, model organisms and *in vitro* systems for the study of LD, neuronal sensitization hypothesis for the etiology of PTLD specifically and neuroborreliosis in general, among others.

In closing, in consideration of the unique global circumstances with the COVID-19 pandemic, it is important to highlight several features of the current context that may impact the LD community. For example, COVID-19 may further complicate diagnosis of LD since non-specific symptoms in these two conditions overlap and people may be spending more time outdoors. The emergence of a persistent syndrome, popularly referred to as long COVID, among a subset of patients following treatment or convalescence (344) may invigorate research and provide insights that carry-over into other infectious diseases with post-treatment sequelae, such as LD.

AUTHOR CONTRIBUTIONS

JRB wrote the first draft of the manuscript. BLJ, EJH, MEE, AB, and RLM wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of Interest: AGR is a scientific advisor to NeonMind Biosciences and ETHA Natural Botanicals. In conjunction with Dr. S. Sontakke and North Carolina State University, EBB holds US Patent No. 7,115,385; Media and Methods for Cultivation of Microorganisms, which was issued on October 3rd, 2006. He is a co-founder, shareholder and Chief Scientific Officer for Galaxy Diagnostics, a company that provides advanced diagnostic testing for the detection of Bartonella spp. and Borrelia infections. RM is a co-founder, shareholder and the Chief Technical Officer for Galaxy Diagnostics. RTM is a paid consultant/speaker for Zoetis and receives license related income from Zoetis. MJS serves on the Scientific Advisory Board for the Global Lyme Alliance and has been issued a patent [US Patent No. 10 481 165] for "Elevated CCL19 after completion of therapy for acute Lyme disease identifies patients at risk for development of post-treatment Lyme disease syndrome who will benefit from further antibiotic therapy". AO has pending patent applications on the development of Lyme disease diagnostic tests.

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