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Immunodiagnosics of Lyme neuroborreliosis

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**AN ENZYME-LINKED IMMUNOSORBENT
SPOT ASSAY MEASURING
BORRELIA BURGDORFERI
B31-SPECIFIC INTERFERON GAMMA-
SECRETING T CELLS CANNOT
DISCRIMINATE ACTIVE LYME
NEUROBORRELIOSIS FROM PAST LYME
BORRELIOSIS: A PROSPECTIVE STUDY IN
THE NETHERLANDS**

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ABSTRACT

Two-tier serology testing is most frequently used for the diagnosis of Lyme borreliosis (LB); however, a positive result is no proof of active disease. To establish a diagnosis of active LB, better diagnostics are needed. Tests investigating the cellular immune system are available, but studies evaluating the utility of these tests on well-defined patient populations are lacking. Therefore, we investigated the utility of an enzyme-linked immunosorbent spot (ELISpot) assay to diagnose active Lyme neuroborreliosis. Peripheral blood mononuclear cells (PBMCs) of various study groups were stimulated by using *Borrelia burgdorferi* strain B31 and various recombinant antigens, and subsequently, the number of *Borrelia*-specific interferon gamma (IFN- γ)-secreting T cells was measured. We included 33 active and 37 treated Lyme neuroborreliosis patients, 28 healthy individuals treated for an early manifestation of LB in the past, and 145 untreated healthy individuals. The median numbers of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs did not differ between active Lyme neuroborreliosis patients (6.0; interquartile range [IQR], 0.5 to 14.0), treated Lyme neuroborreliosis patients (4.5; IQR, 2.0 to 18.6), and treated healthy individuals (7.4; IQR, 2.3 to 14.9) ($P = 1.000$); however, the median number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs among untreated healthy individuals was lower (2.0; IQR, 0.5 to 3.9) ($P < 0.016$). We conclude that the *Borrelia* ELISpot assay, measuring the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs, correlates with exposure to the *Borrelia* bacterium, but cannot be used for the diagnosis of active Lyme neuroborreliosis.

KEYWORDS

Borrelia burgdorferi, ELISpot, Lyme borreliosis, Lyme neuroborreliosis, T-cell activation, active disease, antibodies, cytokines, diagnostics, interferon gamma

In the Netherlands, Lyme borreliosis (LB) poses a considerable threat to human health. A study among general practitioners (GPs) found a threefold increase of patients reporting tick bites and diagnoses of erythema migrans (EM), an early, localized skin rash, in the period between 1994 and 2009 [1]. Between 2009 and 2014, the incidence of reported tick bites ranged between 488 and 564 consultations per 100,000 inhabitants and the number of GP-reported diagnoses of EM ranged between 134 and 140 per 100,000 inhabitants [2]. The true incidence rate is probably higher, since only a small part of the population consults a GP after a tick bite [3]. Increased incidences of LB have also been reported in several other European countries as well as in the United States [4-6].

Diagnosis of active LB can be difficult in the absence of a “gold standard” test, such as PCR or culture. Exceptions are an EM, which is a clinical diagnosis, and acrodermatitis chronica atrophicans (ACA) or Lyme arthritis, which can be supported by PCR and/or culture. For Lyme neuroborreliosis, culture and PCR are too insensitive to be useful in a routine clinical setting [7-10]. The diagnosis of Lyme neuroborreliosis is based on clinical symptoms and needs to be supported by laboratory tests. The most frequently found clinical symptoms of Lyme neuroborreliosis are (lymphocytic) meningoradiculitis and paresis [11]; however, symptoms can be nonspecific, which often complicates the diagnosis. Confirmation of Lyme neuroborreliosis through laboratory testing consists of the detection of *Borrelia*-specific antibodies in cerebrospinal fluid (CSF) and an elevated number of mononuclear cells in CSF, otherwise known as pleocytosis (≥ 5 leukocytes/ μ l) [11]. Unfortunately, studies using well-characterized and unbiased patient groups are rare and the sensitivity and specificity of the various tests can vary extensively [12].

The presence of intrathecally produced *Borrelia*-specific antibodies can indicate active Lyme neuroborreliosis, but also the persistence of *Borrelia*-specific antibodies years after an asymptomatic or treated infection [13]. The absence of *Borrelia*-specific antibodies, on the other hand, does not rule out an active infection and can be explained by the (low) sensitivity of the test used and the time it takes for the body to produce detectable levels of *Borrelia*-specific antibodies after an infection [14]. As early and correct diagnosis of Lyme neuroborreliosis is essential for adequate treatment with antibiotics [15-17], better diagnostic tools are warranted. The diagnostic shortcomings underline the need for new diagnostic tests that can distinguish between active disease and a previous, yet cleared, infection or that can aid in the diagnosis for those cases for which the current diagnostics are insufficient. In this study, active Lyme neuroborreliosis patients were used as a proxy for active disease.

In recent years, assays that focus on the cellular immune response for the diagnosis of LB have become available. The cellular immune response against *Borrelia* is characterized by a strong Th1 response, in which *Borrelia* activates Th1-like cytokines such as interferon gamma (IFN- γ) [18-20]. Elevated amounts of Th1-specific IFN- γ in blood, synovial fluid, and CSF of LB patients have been found in various studies [21-25]. However, compared to serology, T-cell assays were less sensitive and specific, and in general these assays were not well standardized [10, 26]. Despite the lack of published studies on clinically validated cellular assays, various laboratories offer these assays for the diagnosis of LB. These assays include the enzyme-linked immunosorbent spot (ELISpot) assay [27] and the lymphocyte transformation test (LTT) [28]. Therefore, the clinical validation of these assays is urgently needed.

In this study, we performed the validation of a *Borrelia* ELISpot assay measuring the number of IFN- γ -producing T cells after stimulation with *Borrelia burgdorferi* B31. Information regarding previous tick bites, symptoms, and antibiotic treatment for LB was assessed by the completion of a Lyme-specific questionnaire and through consulting electronic patient files. We used a standardized assay on well-defined groups of both treated and untreated patients and healthy

controls to investigate whether the number of *Borrelia*-specific T cells isolated from blood can be used as a marker for disease activity.

MATERIALS AND METHODS

STUDY POPULATION

Whole-blood and serum samples were obtained from hospital patients diagnosed with active Lyme neuroborreliosis, hospital patients treated for Lyme neuroborreliosis in the past, and healthy individuals (all ≥ 18 years old). All hospital patients diagnosed with Lyme neuroborreliosis in Diaconessenhuis Hospital, Utrecht, and St. Antonius Hospital, Nieuwegein, the Netherlands, were eligible for inclusion in the study if they fulfilled at least two criteria for Lyme neuroborreliosis as defined by the European Federation of Neurological Societies (EFNS) [11]. These criteria are (i) the presence of neurological symptoms suggestive of Lyme neuroborreliosis without other obvious explanations, (ii) CSF pleocytosis (≥ 5 leukocytes/ μL), and (iii) *Borrelia*-specific intrathecal antibody production. If all three criteria were met, a case was categorized as a definite Lyme neuroborreliosis case, and if two criteria were met, a case was categorized as a possible Lyme neuroborreliosis case.

Hospital patients either were recently diagnosed with active Lyme neuroborreliosis or had been treated previously for Lyme neuroborreliosis. Active Lyme neuroborreliosis patients were recruited from December 2010 to December 2016 and were included if blood was drawn within 2 months after the start of antibiotic therapy. In addition, active Lyme neuroborreliosis patients could also be included as treated Lyme neuroborreliosis patients. To make sure that enough time had passed between both inclusions, at least 1 year should have passed after they had finished treatment for their Lyme neuroborreliosis disease episode. Treated Lyme neuroborreliosis patients, who had been diagnosed between February 2003 and September 2014, were enrolled from January 2011 to March 2015 and were included at least 4 months after completion of antibiotic therapy for Lyme neuroborreliosis.

Healthy individuals were recruited in the period between February 2013 and December 2015 from personnel of Diaconessenhuis Hospital, Utrecht, St. Antonius Hospital, Nieuwegein, and the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands. Healthy individuals also included Boy Scout patrol leaders, owners of hunting dogs, and recreational runners. All were invited to participate if they pursued recreational activities in high-risk areas for tick bites. In addition, healthy individuals who had received antibiotic treatment for an early manifestation of LB in the past, as they had reported themselves in the Lyme-specific questionnaire, were analyzed as a separate group and are referred to as treated healthy individuals.

All hospital patients and healthy individuals were asked to complete a Lyme-specific questionnaire. This questionnaire included questions on tick bites, the presence of EM, antibiotic treatment for LB, and self-reported complaints at the moment of inclusion and during possible earlier episodes of LB. Information regarding the clinical symptoms, pleocytosis, and intrathecal antibody production during active disease of the Lyme neuroborreliosis patients was extracted from the hospital information system. Healthy individuals were recruited only if they reported no complaints at the time of the inclusion in the study. All study participants gave their informed consent. The regional Medical Research Ethics Committees United approved the study (Nieuwegein, the Netherlands; MEC-U: NL36407.100.11).

ANTIBODY DETECTION IN SERUM AND SERUM-CSF PAIRS

Borrelia-specific serum antibodies were detected using a two-tier serology protocol [29, 30]. The first test used was the C6 enzyme-linked immunosorbent assay (ELISA) (Immunetics, Boston, MA, USA), which is based on a synthetic C6 peptide and is derived from a highly immunogenic part (invariable region 6) of the VlsE (variable major protein-like sequence, expressed) lipoprotein [31].

Equivocal and positive C6 ELISA results were confirmed by using the *recom*Line immunoglobulin M (IgM) and immunoglobulin G (IgG) immunoblot tests (Mikrogen GmbH, Neuried, Germany). The immunoblot strips detect antibodies against *Borrelia burgdorferi sensu stricto*, *Borrelia afzelii*, *Borrelia garinii*, *Borrelia bavariensis*, and *Borrelia spielmanii* by using different recombinant antigens [32]. Each recombinant antigen has a certain value and will be counted when the intensity of the respective band is greater than or equal to the intensity of the cutoff band. The following antigens, with their respective scores, are used: p100 (IgM and IgG, 5 points each), VlsE (IgM and IgG, 5 points each), p58 (IgM and IgG, 4 points each), p41 (IgM and IgG, 1 point each), p39 (IgM, 4 points, and IgG, 5 points), OspA (IgM and IgG, 5 points each), OspC (IgM, 8 points, and IgG, 5 points), and p18 (IgM and IgG, 5 points each). Immunoblot results were recorded as negative (≤ 5 points), equivocal (6 points), or positive (≥ 7 points). Immunoblotting was performed according to the manufacturer's instructions, and the results were recorded with an automated *recom*Scan system using the *recom*Scan software (Mikrogen GmbH). The final immunoblot result was based on a combination of the results of both immunoglobulins: negative when both IgM and IgG were negative, equivocal when at least one of these was equivocal, and positive when at least one of these was positive. When immunoblot confirmation was performed, this result determined the final serology result, independent of an equivocal or positive C6 ELISA result.

Detection of intrathecally produced *Borrelia*-specific antibodies was done using the second-generation IDEIA Lyme neuroborreliosis test (Oxoid, Hampshire, United Kingdom) [33]. Antibody index (AI) scores of ≥ 0.3 were considered positive. The final AI result was based on a combination of the results of both immunoglobulins: negative when the AIs of both IgM and IgG were negative, equivocal when at least one of these was equivocal, and positive when at least one of these was positive.

Both the C6 ELISA and the IDEIA Lyme neuroborreliosis test were performed according to the manufacturer's instructions using a Dynex DS2 automated ELISA instrument (Dynex Technologies), and results were analyzed with DS-Matrix software (Dynex Technologies).

BORRELIA ELISPOT PROCEDURE

The *Borrelia* ELISpot assay was performed on peripheral blood isolated from all study participants. The isolation of peripheral blood mononuclear cells (PBMCs) from whole-blood specimens (lithium heparin) drawn < 8 h before testing was done through density gradient centrifugation (Hettich Rotanta 460 RS; rotor 5624) at room temperature for ~ 15 min at $1,000 \times g$ using Leucosep tubes (OxFord Immunotec Ltd., Abingdon, UK); however, for blood that was drawn between 8 and 32 h before testing, a T-cell Xtend (OxFord Immunotec Ltd.) step was performed following the procedure described by Bouwman et al. [34] prior to PBMC isolation. After centrifugation, the PBMC fraction was removed and washed twice. The first wash step was performed at room temperature for ~ 7 min at $600 \times g$; the second wash step was also performed at room temperature for ~ 7 min at $300 \times g$. Both wash steps were performed in 10 ml of fresh, prewarmed (37°C) RPMI medium (Life Technologies, Invitrogen, Bleiswijk, the Netherlands). If necessary, excess erythrocytes were removed between the first and second wash steps using human erythrocyte lysis buffer (0.010 M KHCO_3 , 0.0001 M EDTA , $0.150 \text{ M NH}_4\text{Cl}$ [$\text{pH } 7.3 \pm 0.1$]). After addition of 5 ml of lysis buffer, the solution was incubated for 5 min at 2°C and subsequently

centrifuged using the first wash step centrifugation program.

The final pellet was suspended in 1.1 ml of fresh, prewarmed (37°C) AIM-V medium (Life Technologies), and cells were counted using the AC.T diff 2 analyzer (Beckman Coulter, Woerden, the Netherlands). Cells were adjusted to 2.5×10^6 /ml and 100 μ l of that concentration was added to a precoated polyvinylidene difluoride (PVDF) ELISpot^{PRO} well (Mabtech, Nacka Strand, Sweden). The negative control consisted of 50 μ l of AIM-V medium, and as a positive control, 50 μ l (0.1 μ g/ml) of anti-human CD3 monoclonal antibody (MAb) CD3-2 (Mabtech) was used. To stimulate the cells, 50 μ l of a whole-cell lysate (5 μ g/ml), a peptide mix (5 μ g/ml), and five recombinant antigens were tested (15 μ g/ml). The whole-cell lysate tested was derived from *B. burgdorferi* strain B31 (Autoimmun Diagnostika GmbH, Straßberg, Germany). The peptide mix (an Osp-mix) consisted of a pool of 9-mer to 11-mer peptides of OspA (*B. burgdorferi*, *B. afzelii*, and *B. garinii*), native OspC (*B. afzelii*), and recombinant p18 (Autoimmun Diagnostika GmbH). The five recombinant antigens used were (i) p18 *B. burgdorferi* sensu stricto PKa, (ii) p18 *B. afzelii* PKo, (iii) p18 *B. garinii* PBi, (iv) p39 *B. afzelii* PKo, and (v) p58 *B. garinii* PBi (Mikrogen GmbH), which are also part of the *recomLine* immunoblot test (Mikrogen GmbH). The number of different antigens tested depended on the yield of PBMCs. After 16 to 20 h at 37°C and 5% CO₂, the wells were washed using phosphate-buffered saline (PBS; pH 7.2 ± 0.1) and incubated for 1 h at 2°C after addition of 50 μ l of 7-B6-1-alkaline phosphatase (ALP) conjugate (Mabtech). The wells were washed again in PBS and incubated with 50 μ l of 5-bromo-4-chloro-3'-indolylphosphate and nitroblue tetrazolium (BCIP/NBT) plus substrate (Mabtech) for ~7 to 10 min at room temperature.

ANALYSIS OF THE BORRELIA ELISPOT ASSAY RESULTS

The number of *Borrelia*-specific IFN- γ -producing T cells, displayed as black spots, was measured with an ELISpot reader (Autoimmun Diagnostika GmbH), visually checked, and, if judged necessary, adjusted manually by two different operators in the EliSpot 6.0 software (Autoimmun Diagnostika GmbH). The spot size used was based on the expected spot size of an IFN- γ -producing T cell as determined by Feske et al. [35] and was set on -2.8 log (mm²). If there was a difference in the T-cell count between the two operators of ≥ 4 spots for a certain sample, or if they found any difference in spot count in the critical area (between 2 and 5 spots), then those samples were recounted by a third operator, whose result was leading.

To determine the actual spot count due to the stimulation of T cells by *Borrelia*, the number of spots in the negative-control well was subtracted from the number of spots in the antigen-stimulated well. The number of spots corresponds with the number of individual T cells producing IFN- γ after antigen stimulation. Different lot numbers of *B. burgdorferi* B31 lysate were used; however, they were derived from the same batch of *B. burgdorferi* B31 lysate. If a blood sample was tested with >1 lot number, or multiple times with an identical lot number, then the median spot count was calculated and used in the *Borrelia* ELISpot analysis.

DATA HANDLING AND STATISTICAL ANALYSIS

For statistical analyses, the IBM SPSS software package (version 23) was used (IBM, Armonk, NY, USA). Dichotomous data were analyzed by using Pearson's chi-square test or Fisher's exact test. The *post-hoc* tests consisted of two-group comparisons by using Pearson's chi-square test or Fisher's exact test using the Bonferroni correction. *P* values of <0.05 were interpreted as statistically significant. If the Bonferroni correction was applied, then a *P* value of 0.05/*k* (for which *k* is the number of different hypotheses) was interpreted as statistically significant. For statistical analyses, equivocal serology results were combined with positive serology results.

Quantitative, unrelated data comparing >2 groups were analyzed by using the Kruskal-Wallis test, and *post-hoc* tests consisted of the Dunn-Bonferroni test. Quantitative, unrelated data

comparing two groups were analyzed using the Mann-Whitney test. Correlations were calculated using Spearman's correlation coefficient (r_s). Quantitative, related data comparing >2 tests were analyzed using Friedman's related-samples two-way analysis of variance test, and *post-hoc* tests consisted of the Dunn-Bonferroni test. Quantitative, related data, comparing two tests, were analyzed using the Wilcoxon signed-rank test. For all analyses, *P* values of <0.05 were interpreted as statistically significant.

To determine the utility of the *Borrelia* ELISpot assay to diagnose active Lyme neuroborreliosis, a receiver operating characteristic (ROC) curve was created to calculate the area under the curve (AUC). Therefore, the number of *Borrelia*-specific IFN- γ -secreting T cells among active Lyme neuroborreliosis patients was compared with the number of *Borrelia*-specific IFN- γ -secreting T cells among the other three groups. Logistic regression was applied to investigate whether any additional risk factors could contribute to the diagnostic performance of the *Borrelia* ELISpot assay. The Hosmer-Lemeshow goodness-of-fit test was used to assess if the logistic regression model fit the data. The outcome of the model was binary: a case was either an active patient or a control. Figures were made with GraphPad Prism (version 5.04 for Windows; GraphPad Software, San Diego, CA, USA).

RESULTS

STUDY POPULATION

ACTIVE LYME NEUROBORRELIOSIS PATIENTS

Thirty-three active Lyme neuroborreliosis patients were included; their median age was 56.7 years (interquartile range [IQR], 44.8 to 64.4 years). They were included before, during, or shortly after antibiotic treatment started (median, 7.0 days after the start of antibiotic therapy; IQR, 3.0 to 12.5 days) (Table 1). Antibiotic therapy consisted of intravenous ceftriaxone for 14 or 30 days. Two patients switched to doxycycline because of an adverse reaction to ceftriaxone. One patient was given doxycycline from the start (21 days). The clinical symptoms among active Lyme neuroborreliosis patients mostly consisted of radicular disease (15/33 [45.5%]) and/or cranial nerve paresis (15/33 [45.5%]).

The majority of the active Lyme neuroborreliosis patients, 25/33 (75.8%), were classified as definite Lyme neuroborreliosis patients and 8/33 (24.2%) of them as possible Lyme neuroborreliosis patients, because they lacked production of intrathecal antibody against *Borrelia* (Table 2). Only three patients had a positive antibody index (AI) based on a solitary IgM response, 12 patients had positive AIs for both IgM and IgG, and 10 patients had a positive AI based on a solitary IgG response (see Table S1 in the supplemental material).

Table 1. Demographic and clinical characteristics of the four study groups^a

Variable	Active Lyme NB patients ^b (n = 33)	Treated Lyme NB patients ^b (n = 37)	Treated healthy individuals (n = 28)	Untreated healthy individuals (n = 145)	P value	
					Overall	2-group ^c
Males (n; %)	22 (66.7)	19 (51.4)	13 (46.4)	55 (37.9)	0.020	0.003 ^k
Median age, yrs (IQR)	56.7 (44.8-64.4)	59.3 (49.4-66.9)	52.7 (38.1-57.5)	41.0 (27.0-51.7)	<0.001	≤0.029 ^l
Tick bite (n; %)	11 (45.8) ^d	27 (73.0)	26 (92.9)	87 (60.0)	0.001	≤0.041 ^m
EM (n; %)	4 (16.7) ^{d,e}	9 (24.3) ^e	22 (78.6) ^h	4 (2.8) ^j	<0.001	≤0.015 ⁿ
No. of positives in two-tier serology testing (%)	30 (90.9)	6 (16.7) ^g	5 (17.9)	18 (12.4)	<0.001	<0.001 ^o
IgM (n; %)	16 (48.5)	3 (8.3)	2 (7.1)	1 (0.7)	<0.001	≤0.025 ^p
IgG (n; %)	28 (84.8)	6 (16.7)	3 (10.7)	18 (12.4)	<0.001	<0.001 ^q
Median time between end of AB and blood sampling, yrs (IQR)	NA	5.0 (2.5 - 7.3)	5 (2 - 7) ⁱ	NA	NA	0.563
Median time between start of AB and blood sampling, days (IQR)	7.0 (3.0-12.5)	NA	NA	NA	NA	NA
Self-reported complaints at inclusion	Table 2 ^f	23 (62.2%)	0	0	NA	NA

- a. EM, erythema migrans; AB, antibiotic treatment for Lyme borreliosis; IQR, interquartile range; NB, neuroborreliosis; n, number of study participants; NA, not applicable.
- b. Six active Lyme neuroborreliosis patients were also included as treated neuroborreliosis patients (>1 year after they had finished treatment for their Lyme neuroborreliosis disease episode).
- c. For all two-group comparisons with a significant difference, the Bonferroni correction was applied.
- d. Nine (27.3%) active Lyme neuroborreliosis patients did not complete the Lyme-specific questionnaire, so data on tick bite and/or EM were not present for them.
- e. One patient with erythema migrans did not recall a tick bite; all others did recall a tick bite.
- f. For active Lyme neuroborreliosis patients, instead of the self-reported complaints, we assessed the electronic patients files for clinical symptoms due to Lyme neuroborreliosis. Those symptoms are listed in Table 2.
- g. For one (2.7%) treated Lyme neuroborreliosis patient, two-tier serology testing was not done because of the lack of a serum sample.
- h. Two individuals with erythema migrans did not recall a tick bite and six individuals did not report an erythema migrans and were treated for an atypical skin rash (n = 4), flu-like symptoms after the tick bite (n = 1), or the presence of an engorged adult tick (n = 1).
- i. One (3.6%) individual who did not know when antibiotic treatment took place was excluded.
- j. All individuals with erythema migrans recalled a tick bite.
- k. Untreated healthy individuals versus active Lyme neuroborreliosis patients.
- l. Untreated healthy individuals versus all other groups.
- m. Treated healthy individuals versus all other groups (P ≤ 0.041); treated Lyme neuroborreliosis patients versus active Lyme neuroborreliosis patients (P = 0.033).
- n. Treated healthy individuals versus all other groups (P < 0.001); untreated healthy individuals versus both Lyme neuroborreliosis patient groups (P ≤ 0.015).
- o. Active Lyme neuroborreliosis patients versus all other groups.
- p. Active Lyme neuroborreliosis patients versus all other groups (P < 0.001); untreated healthy individuals versus treated Lyme neuroborreliosis patients (P = 0.025).
- q. Active Lyme neuroborreliosis patients versus all other groups.

Table 2. Clinical symptoms and case definitions based on the EFNS criteria [11] of treated and active Lyme neuroborreliosis patients in this study during their active disease period^a

Active Lyme NB patients (n = 33)	Treated Lyme NB patients (n = 37)	Clinical symptoms			Median CSF leucocyte count during diagnosis (/μl) (IQR)	Intrathecal antibody production ^c	EFNS criterion	
		Radicular disease ^b	Cranial nerve paresis	Other			Possible Lyme NB	Definite Lyme NB
8 ^d		x			56.5 (27.3-232.3)	x		x
2		x			131.8 (121.8-141.9)		x	
6			x		158.3 (82.7-254.5)	x		x
4			x		21.0 (17.3-67.5)		x	
2				x ^g	395.5 (304.8-486.3)	x		x
2				x ^g	94.2 (82.6-105.8)		x	
3 ^e		x	x		80.0 (46.2-249.0)	x		x
1		x		x ^h	13.3	x		x
1 ^f		x	x	x ^g	473.7	x		x
1			x	x ^g	377.3	x		x
1				x ⁱ	41.0	x		x
Median pleocytosis (IQR)					111.7 (21.0-243.5)			
Total (n; %)		15 (45.5)	15 (45.5)	10 (30.3)		25 ^k (75.8)	8 (24.2)	25 (75.8)
	15 ^d	x			50.0 (32.5-105.8)	x		x
	2	x			<5	x	x	
	7		x		60.0 (44.0-83.5)	x		x
	2		x		<5	x	x	
	5 ^e	x	x		88.0 (20.3-128.0)	x		x
	1	x	x		76.0		x	
	1 ^f	x	x	x ^g	473.7	x		x
	1		x	x ^h	83.3	x		x
	2			x ⁱ	94.8 (74.3-115.4)	x		x
	1			x ^h	<5	x	x	
Median pleocytosis (IQR)					52.0 (22.2-105.9)			
Total (n; %)		24 (64.9)	17 (45.9)	5 (13.5)		36 ^{l,m} (97.3)	6 (16.2)	31 (83.8)
P value					0.071	0.010	0.402 ⁿ	

a. CSF, cerebrospinal fluid; EFNS, European Federation of Neurological Societies.

b. Radicular disease was based on either radiculopathy, radiculitis, or radiculomyelitis.

c. Detailed information regarding the antibody index for active Lyme neuroborreliosis patients can be found in Table S1.

d. Four patients were included as active and treated Lyme neuroborreliosis patients.

e. One patient was included as an active and treated Lyme neuroborreliosis patient.

f. The patient was included as an active and treated Lyme neuroborreliosis patient.

g. Patient diagnosed with meningitis.

h. Patient diagnosed with peripheral neuropathy.

i. One patient diagnosed with encephalitis, one patient diagnosed with a cerebrovascular accident, and one patient diagnosed with peripheral neuropathy.

j. One patient diagnosed with meningitis and one patient diagnosed with peripheral neuropathy.

k. Sixteen (48.5%) patients had a positive IgM antibody index, and 22 (66.7%) patients had a positive IgG antibody index. For one patient the IgM antibody index could not be determined.

l. Seventeen (45.9%) patients had a positive IgM antibody index, and 36 (97.3%) had a positive IgG antibody index; see also Table S1.

m. A significantly higher number of treated Lyme neuroborreliosis patients had intrathecal antibody production at the time they were diagnosed with active Lyme neuroborreliosis than in the active Lyme neuroborreliosis patient group.

n. No difference was found in EFNS criteria between active Lyme neuroborreliosis patients and treated Lyme neuroborreliosis patients.

TREATED LYME NEUROBORRELIOSIS PATIENTS

Thirty-seven Lyme neuroborreliosis patients were included at a median of 5.0 years (IQR, 2.5 to 7.3 years) after they had finished antibiotic therapy for LB (Table 1). The median age of the treated Lyme neuroborreliosis patients at inclusion was 59.3 years (IQR, 49.4 to 66.9 years) (Table 1). Antibiotic therapy consisted of intravenous ceftriaxone for 14 or 30 days; one patient switched to doxycycline (for 14 days) due to an allergic reaction to ceftriaxone. Most treated Lyme neuroborreliosis patients suffered from radicular disease (24/37 [64.9%]) and/or cranial nerve paresis (17/37 [45.9%]), which was similar to what was observed for the active Lyme neuroborreliosis patients (Table 2).

Thirty-one (83.8%) out of the 37 treated Lyme neuroborreliosis patients were, when they were diagnosed with active Lyme neuroborreliosis in the past, classified as definite Lyme neuroborreliosis patients and 6/37 (16.2%) as possible Lyme neuroborreliosis patients, of whom the majority did not have pleocytosis (5/6 [83.3%]) (Table 2). This was in contrast with the active Lyme neuroborreliosis patients, who were, when diagnosed with active Lyme neuroborreliosis, more often classified as possible Lyme neuroborreliosis patients, because of the absence of intrathecal produced *Borrelia*-specific antibodies ($P = 0.010$) (Table 2). A total of 36 (97.3%) of the 37 treated Lyme neuroborreliosis patients had a positive AI for IgG, of whom 17 (47.2%) also had a positive AI for IgM (data not shown). Interestingly, 23/37 (62.2%) of the treated Lyme neuroborreliosis patients still reported complaints when they were included in this study (Table 1). These self-reported complaints included neuropathic complaints, cognitive complaints, fatigue, myalgias, paraesthesias, and/or malaise. A total of six treated Lyme neuroborreliosis patients had also been included as active Lyme neuroborreliosis patients at the time they were diagnosed with active Lyme neuroborreliosis; the median time between the end of antibiotic treatment for Lyme neuroborreliosis and inclusion in this study as a treated Lyme neuroborreliosis patient was 2.3 years (IQR, 1.6 to 2.7 years).

HEALTHY INDIVIDUALS

One hundred seventy-three healthy individuals were included; their median age at inclusion was 42.2 years (IQR, 27.5 to 53.2 years). Twenty-eight (16.2%) out of these individuals reported antibiotic therapy for LB in the past (median, 5 years ago; IQR, 2 to 7 years), and they were classified separately as treated healthy individuals (Table 1). Most treated healthy individuals reported antibiotic treatment for EM (22/28 [78.6%]); the six remaining individuals were treated for an atypical skin rash ($n = 4$), flu-like symptoms after the tick bite ($n = 1$), or the presence of an engorged adult tick ($n = 1$). The remaining 145 (83.8%) healthy individuals were classified as untreated healthy individuals. The median age of the treated healthy individuals was 52.7 years (IQR, 38.1 to 57.5 years). In this group, the percentage of tick bites was higher than in all other groups ($P \leq 0.041$). Comparison of the four study groups showed that the percentage of EM was also highest among treated healthy individuals (22/28 [78.6%]) ($P \leq 0.001$) (Table 1). The untreated healthy individuals were younger than the other three groups (median, 41.0 years; IQR, 27.0 to 51.7 years) ($P \leq 0.029$), and 87/145 (60.0%) recalled a tick bite; four of them also mentioned an EM. The percentage of reported EM within this group was lower than for all other groups ($P \leq 0.015$) (Table 1).

TWO-TIER SEROLOGY RESULTS

Serology testing showed that most of the active Lyme neuroborreliosis patients were seropositive (30/33 [90.9%]) (Table 1). Twenty-eight (84.8%) of the 33 seropositive patients had IgG antibodies; 16/33 (48.5%) also had IgM antibodies (Table 1). Only two active Lyme neuroborreliosis patients had a positive serology result based on a solitary IgM response. Both IgM and IgG were more often found among active Lyme neuroborreliosis patients than among the other three groups ($P < 0.001$ for both) (Table 1).

For 36/37 treated Lyme neuroborreliosis patients, a serum sample was available for serology; 6/36 (16.7%) had a positive result (Table 1). For all six cases, the result was based on an IgG response; three of them also had an IgM response (Table 1). Interestingly, no difference was found in serology among treated Lyme neuroborreliosis patients with and without complaints ($P = 1.000$; data not shown). Only 4 (17.4%) out of the 23 treated Lyme neuroborreliosis patients with complaints had *Borrelia*-specific antibodies, and 2 (15.4%) out of the 13 treated Lyme neuroborreliosis patients without complaints were seropositive (the serum of one patient without complaints was missing). The two-tier serology of the six patients that were included both as an active Lyme neuroborreliosis patient and later as a treated Lyme neuroborreliosis patient showed that four (66.7%) out of the six patients reverted from seropositive to seronegative. These six patients all had Lyme neuroborreliosis-specific symptoms at the time of their diagnosis with active disease (all had radicular disease; two had facial nerve paresis as well, of whom one also had meningitis). These Lyme neuroborreliosis-specific symptoms had all disappeared at the time they were included in the study as a treated Lyme neuroborreliosis patient. Two (33.3%) of them did not report any complaints at all, but four (66.7%) reported nonspecific symptoms, such as fatigue ($n = 2$), loss of focus and/or amnesia ($n = 2$), loss of strength ($n = 1$), early-onset rheumatoid arthritis ($n = 1$), urinary problems ($n = 1$), and arrhythmia ($n = 1$).

Among the 173 healthy individuals, a total of 23 (13.3%) had *Borrelia*-specific serum antibodies. Positive serology results were found among both treated and untreated healthy individuals (5/28 [17.9%] and 18/145 [12.4%], respectively) (Table 1). Among treated healthy individuals, positive serology results were based on either an IgG response (3/5 [60.0%]) or an IgM response (2/5 [40.0%]). Among untreated healthy individuals, all 18 positive serology results were based on an IgG response; only 1 (5.6%) of them also had an IgM response (Table 1). All 23 healthy individuals with a positive serology result were invited to consult an infectious diseases specialist, and 17 (73.9%) of them did indeed visit the specialist. None of them had any signs or symptoms suggesting a current or recent (symptomatic) LB.

PERFORMANCE OF THE *BORRELIA* ELISPOT ASSAY WITH DIFFERENT *BORRELIA* ANTIGENS

Analysis of the final spot counts of both operators showed that one operator systematically had higher spot counts. The correlation between both operators, however, was very high (r_s 0.913; $P < 0.001$).

All 243 study participants were tested with *B. burgdorferi* B31 whole-cell lysate; a subset of study participants was also tested with the other *Borrelia* antigens (Table 3). In general, stimulation with Osp-mix resulted in fewer IFN- γ -secreting T cells/ 2.5×10^5 PBMCs than stimulation with *B. burgdorferi* B31 ($P < 0.001$ [all study participants] and $P \leq 0.028$ [within the study groups]) (Table 3). A similar trend was seen for the different recombinant antigens compared to *B. burgdorferi* B31 ($P < 0.001$ [all study participants] and $P \leq 0.020$ [within the study groups]) (Table 3). Interestingly, only for active Lyme neuroborreliosis patients was an association found between the number of Osp-mix-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs and the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs (r_s 0.723; $P < 0.001$; $n = 21$) (data not shown). Since the *B. burgdorferi* B31 lysate resulted in the highest number of IFN- γ -secreting T cells/ 2.5×10^5 PBMCs, those results were used in all further (statistical) analyses.

Table 3. Various *Borrelia* antigens used for stimulating T cells in the *Borrelia* ELISpot

Study groups	Parameter	<i>B. burgdorferi</i> B31	Recombinant antigens					<i>B. burgdorferi</i> B31 vs Osp- mix ^b P value	<i>B. burgdorferi</i> B31 vs recombinant antigen P value
			Osp-mix ^b	<i>B. burgdorferi</i> p18 PKa	<i>B. afzelii</i> p18 PKo	<i>B. garinii</i> p18 PBi	<i>B. afzelii</i> p39 PKo	<i>B. garinii</i> p58 PBi	
All study participants	<i>n</i>	243	95	119	118	118	118	118	
	%	100	39.1	49.0	48.6	48.6	48.6	48.6	
<i>Borrelia</i> ELISpot result ^a		3.0	1.0	0.0	0.0	0.0	0.0	1.0	<0.001 ^e
IQR		1.0-6.5	0.0-2.5	0.0-1.0	0.0-1.1	0.0-1.0	0.0-2.0	0.0-2.6	<0.001 ^e
Active Lyme NB patients	<i>n</i>	33	21	16	16	16	16	16	
	%	100	63.6	48.5	48.5	48.5	48.5	48.5	
<i>Borrelia</i> ELISpot result ^a		6.0	1.0	0.0	0.0	0.0	0.0	1.5	<0.001 ^e
IQR		0.5-14.0	0.0-4.5	0.0-0.8	0.0	0.0-1.8	0.0-1.4	1.0-4.9	<0.001 ^e
Treated Lyme NB patients	<i>n</i>	37	13	22	22	22	22	22	
	%	100	35.1	59.5	59.5	59.5	59.5	59.5	
<i>Borrelia</i> ELISpot result ^a		4.5	0.0	0.0	0.0	0.5	0.0	2.8	<0.001 ^e
IQR		2.0-18.6	0.0-5.0	0.0-1.0	0.0-2.6	0.0-2.0	0.0-2.1	0.0-5.3	<0.001 ^e
Treated healthy individuals	<i>n</i>	28	12	14	14	14	14	14	
	%	100	42.9	50.0	50.0	50.0	50.0	50.0	
<i>Borrelia</i> ELISpot result ^a		7.4	2.0	0.0	1.0	0.0	1.0	1.0	<0.001 ^e
IQR		2.3-14.9	0.3-6.0	0.0-1.0	0.0-2.3	0.0-2.0	0.0-6.0	0.0-3.3	<0.001 ^e

Table 3. Continued.

Study groups	Parameter	<i>B. burgdorferi</i> B31	Osp-mix ^b	Recombinant antigens				<i>B. burgdorferi</i> B31 vs Osp- mix ^b		<i>B. burgdorferi</i> B31 vs recombinant antigen <i>P</i> value
				<i>B. burgdorferi</i> p18 PKa	<i>B. afzelii</i> p18 PKo	<i>B. garinii</i> p18 PBi	<i>B. afzelii</i> p39 PKo	<i>B. garinii</i> p58 PBi	<i>P</i> value	
Untreated healthy individuals	<i>n</i>	145	49	67	66	66	66	66	Overall	2-group
	%	100	33.8	46.2	45.5	45.5	45.5	45.5		
	<i>Borrelia</i> ELISpot result ^a	2	1	0	0	0	0	0	0.017 ^e	<0.001 ^f
	IQR	0.5-3.9	0-1.5	0-1.0	0-1.0	0-1.0	0-1.6	0-1.0		
<i>P</i> value (overall)		<0.001	0.227	0.735	0.311	0.943	0.219	0.002		
<i>P</i> value (2-group)		≤0.016 ^c	NC ^k	NC	NC	NC	NC	≤0.004 ^d		

- a. The *Borrelia* ELISpot assay result reflects the median count of the number of activated T cells by the corresponding antigen used/2.5 x 10⁵ peripheral blood mononuclear cells.
- b. The Osp-mix consists of a pool of 9-mer to 11-mer peptides of OspA (*B. burgdorferi*), *B. afzelii*, and *B. garinii*), native OspC (*B. afzelii*), and recombinant p18.
- c. Untreated healthy individuals had significantly lower numbers of *B. burgdorferi* B31-specific IFN-γ-secreting T cells/2.5 x 10⁵ peripheral blood mononuclear cells than both treated groups (*P* < 0.001) and active Lyme neuroborreliosis patients (*P* = 0.016) (see also Fig. 1).
- d. Untreated healthy individuals had significantly lower numbers of *B. garinii* p58 PBi-specific IFN-γ-secreting T cells/2.5 x 10⁵ peripheral blood mononuclear cells than active Lyme neuroborreliosis patients.
- e. Among all study participants, stimulation with Osp-mix resulted in significantly lower numbers of activated T cells/2.5 x 10⁵ peripheral blood mononuclear cells than stimulation with *B. burgdorferi* B31 (*P* < 0.001). This was also found among the four study groups separately (*P* = 0.001 to 0.028).
- f. For all five recombinant antigens, stimulation resulted in significantly lower numbers of activated T cells/2.5 x 10⁵ peripheral blood mononuclear cells than stimulation with *B. burgdorferi* B31 (*P* < 0.001). Stimulation with *B. burgdorferi* B31 PKa also resulted in a lower number of activated T cells/2.5 x 10⁵ peripheral blood mononuclear cells than stimulation with *B. garinii* p58 PBi (*P* = 0.047).
- g. For two recombinant antigens, *B. burgdorferi* p18 PKa and *B. afzelii* p18 PKo, stimulation resulted in significantly lower numbers of activated T cells/2.5 x 10⁵ peripheral blood mononuclear cells than stimulation with *B. burgdorferi* B31 (*P* < 0.020) or stimulation with *B. garinii* p58 PBi (*P* < 0.017).
- h. For four recombinant antigens, stimulation resulted in significantly lower numbers of activated T cells/2.5 x 10⁵ peripheral blood mononuclear cells than stimulation with *B. burgdorferi* B31; the exception was *B. garinii* p58 PBi.
- i. For four recombinant antigens, stimulation resulted in significantly lower numbers of activated T cells/2.5 x 10⁵ peripheral blood mononuclear cells than stimulation with *B. burgdorferi* B31; the exception was *B. afzelii* p39 PKo.
- j. For all recombinant antigens, stimulation resulted in significantly lower numbers of activated T cells/2.5 x 10⁵ peripheral blood mononuclear cells than stimulation with *B. burgdorferi* B31.
- k. NC, not calculated.

BORRELIA ELISPOT ASSAY RESULTS BY STUDY GROUP AND SELF-REPORTED COMPLAINTS

No significant difference was found in the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs between the following three groups: active Lyme neuroborreliosis patients (median, 6.0; IQR, 0.5 to 14.0), treated Lyme neuroborreliosis patients (median, 4.5; IQR, 2.0 to 18.6), and treated healthy individuals (median, 7.4; IQR, 2.3 to 14.9) ($P = 1.000$) (Fig. 1). However, these three groups had higher numbers of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs than untreated healthy individuals (median, 2.0; IQR, 0.5 to 3.9) ($P \leq 0.016$) (Table 3).

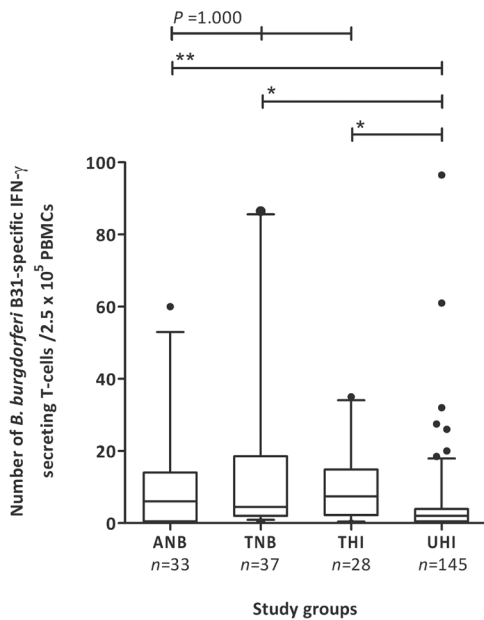


Fig. 1. *B. burgdorferi* B31-specific T-cell activation among active Lyme neuroborreliosis patients, treated Lyme neuroborreliosis patients, treated healthy individuals, and untreated healthy individuals. ANB, active Lyme neuroborreliosis patients; TNB, treated Lyme neuroborreliosis patients; THI, treated healthy individuals; UHI, untreated healthy individuals; n, number of study participants. *, significant difference based on a P value of <0.001 ; **, significant difference based on a P value of 0.016.

More than 60% of the treated Lyme neuroborreliosis patients reported one or more symptoms in the Lyme-specific questionnaire (Table 1); however, no correlation was found between these self-reported symptoms and the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs (r_s , 0.200; $P = 0.235$). The reactivity found among treated healthy individuals also could not be linked to symptomatic disease, since none of the healthy individuals reported any complaints.

DIAGNOSTIC PERFORMANCE OF THE BORRELIA ELISPOT ASSAY

To assess the diagnostic performance of the *Borrelia* ELISpot assay for detecting active Lyme neuroborreliosis, we used a logistic regression model. In the first model, only the results of the *Borrelia* ELISpot assay were used. The outcome of the model was used to create a receiver operating characteristic (ROC) curve. Unfortunately, the area under the curve (AUC) found was only slightly better than a random predictor (model 1; AUC, 0.591) (Fig. 2) and the model did not fit the data ($P = 0.026$) (see Table S2 in the supplemental material).

To determine the (added) value of various risk factors, we also investigated other logistic regression models. The following risk factors were assessed: sex, tick bite, EM, and age (see Table S2). Interestingly, when a model was created for which only the risk factors tick bite and age were included, and thus without the results of the *Borrelia* ELISpot assay, a better AUC was achieved (model 2; AUC, 0.689) (Fig. 2; see also Table S2). Addition of the *Borrelia* ELISpot assay results to the risk factors of model 2 only minimally increased the AUC (model 3; AUC, 0.694) (Fig. 2; see also Table S2). When all risk factors were included in the model, an AUC of 0.741 was found (model 4). Taking into account all possible interaction effects, only “age by *Borrelia* ELISpot” was significant ($P = 0.018$), and adding this to model 4 resulted in an AUC of 0.769 (model 5) (Fig. 2; see also Table S2). In the last model, the absence of a tick bite increased the odds of being an active Lyme neuroborreliosis patient (odds ratio [OR], 2.938; $P = 0.029$). The contribution of the *Borrelia* ELISpot assay result (OR, 1.218; $P = 0.010$), age (OR, 1.061; $P = 0.001$), and the interaction term “age by *Borrelia* ELISpot” (OR, 0.996; $P = 0.018$) was minimal in this model (see Table S2).

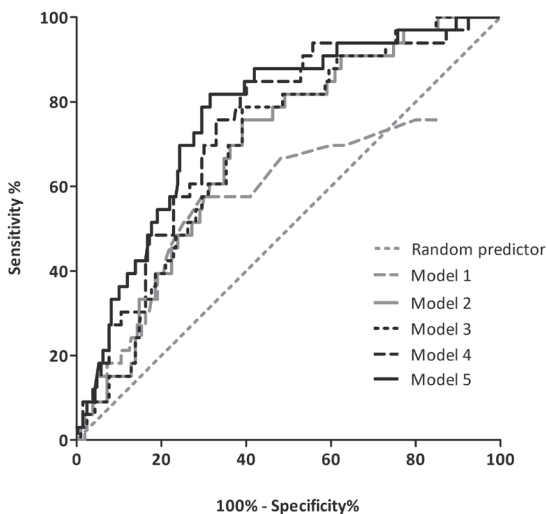


Fig. 2. Receiver operating characteristic (ROC) curve of the *Borrelia* ELISpot assay results and selected logistic regression models that improved the diagnostic performance of the *Borrelia* ELISpot assay used in this study. The ROC curve of model 1 is based on the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs (the *Borrelia* ELISpot assay). Model 2 is based solely on the risk factors tick bite and age and thus is without the addition of the *Borrelia* ELISpot assay results. Model 3 is based on model 2, with the addition of the *Borrelia* ELISpot assay results. Model 4 is based on all risk factors analyzed in this study (i.e., sex, tick bite, EM, and age, in addition to the *Borrelia* ELISpot assay results), and model 5 is based on model 4 with the addition of the interaction term “age by *Borrelia* ELISpot” (see also Table S2).

BORRELIA ELISPOT ASSAY VERSUS TWO-TIER SEROLOGY

In general, seropositive cases had a higher number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs (median, 5.0; IQR, 1.5 to 14.0) than seronegative cases (median, 2.0; IQR, 1.0 to 5.0) ($P = 0.005$) (Table 4). When the four study groups were analyzed separately, no significant difference was found in the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs between seropositive and seronegative cases ($P = 0.070$ to 1.000) (Table 4). Interestingly, among seronegative study participants, less *B. burgdorferi* B31-specific T-cell activity was found among untreated healthy individuals than in both treated groups ($P \leq 0.001$) (data not shown); no difference was found among the seropositive study participants between the four study groups ($P = 0.216$) (data not shown). Analysis of the C6 ELISA index scores, which are semiquantitative, showed an association between the level of the C6 ELISA index scores and

the number of *Borrelia*-specific T cells (r_s , 0.187; $P = 0.004$); however, no association was found within any of the four groups (data not shown).

Table 4. Overview of the number of *B. burgdorferi* B31-specific IFN- γ secreting T-cells among study participants with and without *Borrelia*-specific serum antibodies

Group	Serology result	n	No. of <i>B. burgdorferi</i> B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs		P value
			Median	IQR	
All combined	-	183	2.0	1.0-5.0	0.005 ^b
	+	59	5.0	1.5-14.0	
Active Lyme NB patients	-	3	5.0	2.5-19.5	1.000
	+	30	6.0	0.8-13.5	
Treated Lyme NB patients ^a	-	30	5.5	2.0-18.1	0.664
	+	6	4.3	3.5-36.6	
Treated healthy individuals	-	23	6.0	1.5-14.0	0.121
	+	5	15.0	4.0-34.0	
Untreated healthy individuals	-	127	1.5	0.5-3.8	0.070
	+	18	3.0	1.0-6.3	

- a. For one treated Lyme neuroborreliosis patient, two-tier serology testing was not done because of the lack of a serum sample.
- b. Seronegative study participants had significantly lower numbers of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 peripheral blood mononuclear cells than seropositive study participants.

BORRELIA ELISPOT ASSAY VERSUS ANTIBODY INDEX FOR ACTIVE LYME NEURO-BORRELIOSIS PATIENTS

Because of the prerequisite of a CSF sample to determine the antibody index, AIs were determined only for Lyme neuroborreliosis patients at the time of diagnosis and thus were lacking for the healthy individuals. Only for active cases were the AI and *Borrelia* ELISpot assay results from samples from the same time period available and thus comparable and could be used in subsequent analyses. No difference was found among active Lyme neuroborreliosis patients when the numbers of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs were compared between AI-positive and AI-negative cases ($P = 0.550$) (see Table S1). Similarly, no difference was found among the active patients when the numbers of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs were compared between negative and positive IgM AI results or between negative and positive IgG AI results ($P = 0.081$ and 0.336 , respectively) (see Table S1). The lack of an association between the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs and the level of AI scores of both IgM and IgG was confirmed using Spearman's correlation coefficient (r_s , 0.109 and $P = 0.575$ for IgM and r_s , -0.054 and $P = 0.764$ for IgG). We did, however, find a negative correlation between the level of the AI score for IgM and the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs when only those active patients who had a positive AI for IgM were analyzed ($n = 12$; r_s , -0.694; $P = 0.012$). No such association was found for IgG (data not shown).

DISCUSSION

In this study, we used well-defined patient populations and healthy controls to evaluate the utility of the *Borrelia* ELISpot assay. We found that the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs in peripheral blood was significantly elevated in active Lyme neuroborreliosis patients, treated Lyme neuroborreliosis patients, and healthy individuals treated for early manifestations of LB in the past compared to untreated healthy individuals (P

≤ 0.016). Thus, positive *Borrelia* ELISpot assay results are, in general, associated with exposure and/or (past) infection with *B. burgdorferi* sensu lato. The diagnostic performance of the *Borrelia* ELISpot assay for the detection of active disease was determined by calculation of the ROC curve, which resulted in an AUC of 0.591, suggesting that this assay is unsuitable for the diagnosis of active Lyme neuroborreliosis.

To diagnose Lyme neuroborreliosis, laboratories often rely upon the detection of intrathecally produced *Borrelia*-specific antibodies. The *Borrelia* ELISpot assay, however, did not outperform the AI assay, as the *Borrelia* ELISpot assay results among active Lyme neuroborreliosis patients did not differ between AI-positive and AI-negative patients ($P = 0.550$). We did, however, find a negative correlation among positive AI scores for IgM and the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs in peripheral blood (r_s , -0.694; $P = 0.012$). This is in line with the results found by Dattwyler et al. [36, 37], who showed that a *Borrelia*-specific T-cell response precedes the development of a measurable antibody response.

In this study, we found that a whole-cell lysate of *B. burgdorferi* B31 yielded more activated T cells when used to stimulate the PBMCs than when various recombinant antigens were used for PBMC stimulation. This could be explained by the higher number of antigens present in whole-cell lysates and, hence, more antigenic determinants that can elicit an immune response than the (limited) number of antigenic determinants present among the recombinant antigens used. von Baehr et al. [38] also reported that a whole-cell lysate stimulated PBMCs better than recombinant antigens. We cannot, however, exclude the possibility that the amount of recombinant antigens we used was too low.

We found *Borrelia* ELISpot assay reactivity among treated Lyme neuroborreliosis patients and treated healthy individuals, but this could not be linked to symptoms, although more than 60% of the treated Lyme neuroborreliosis patients in this study still reported (nonspecific) symptoms. Similar percentages have been found in other studies [39-41]. The nonspecific symptoms reported among treated Lyme neuroborreliosis patients could not be linked to the *Borrelia* ELISpot assay results. The *Borrelia* ELISpot assay reactivity among the treated healthy individuals could also not be linked to complaints, as these individuals were included in the study only when they reported having no complaints at all. Therefore, we conclude that the *Borrelia* ELISpot assay reactivity among both treated groups is most likely explained by a previous, cured LB.

Borrelia ELISpot assay reactivity was also found among untreated healthy individuals. Ekerfelt et al. [42] also found elevated numbers of *Borrelia*-specific IFN- γ -secreting T cells in both clinical LB cases and asymptomatic (seropositive) controls after stimulation with an outer surface-enriched fraction of *B. afzelii*. In our study, the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs did not differ between seropositive and seronegative untreated healthy individuals ($P = 0.070$). This could be explained by the low number of seropositive cases (18 seropositive cases versus 127 seronegative cases), although Dattwyler et al. [43] did report an increased T-cell proliferative response to whole-cell *B. burgdorferi* among active LB patients who did not have *Borrelia*-specific antibodies. *Borrelia* ELISpot assay reactivity among untreated, seronegative healthy individuals could also be explained by the choice of the antigen. It is known that the use of whole-cell lysates increases the chance of cross-reactivity, which could lead to false-positive results. The *B. burgdorferi* B31 lysate used contains antigens such as flagellin, which shows high homology with antigens from *Treponema pallidum* or bacteria of the genus *Leptospira*. To investigate for possible cross-reactivity, we also tested the blood of some patients with active neurosyphilis ($n = 2$) and active leptospirosis ($n = 2$), and a strong ELISpot assay reactivity against *B. burgdorferi* B31 was found for one leptospirosis case, but the patients with neurosyphilis did not show *Borrelia* ELISpot assay reactivity (data not shown). Lipopolysaccharides (LPS) present in

whole-cell lysates could also potentially stimulate the T cells, although Janský et al. [18] showed that LPS from *Escherichia coli* did not result in elevated IFN- γ levels and that *Borrelia* lysates did. Other studies showed the production of IFN- γ by NK cells after stimulation with LPS [44, 45]; however, we tried to correct for this phenomenon by adjusting the ELISpot assay reader settings in which we omitted small- and low-intensity spots [35, 46].

Apart from the possible false-positive results, false-negative results were also found, as samples from some active Lyme neuroborreliosis patients did not show *Borrelia* ELISpot assay reactivity. This could be explained by the type of species used to stimulate the T cells, since *B. garinii* and *B. bavariensis* have been linked to Lyme neuroborreliosis more often than *B. burgdorferi*, and *B. afzelii* has been isolated from CSF of Lyme neuroborreliosis patients as well [47, 48]. Since all aforementioned *Borrelia* species are closely related and share many antigens, they will most likely be cross-reactive when used in the *Borrelia* ELISpot assay. Therefore, we decided to test the *B. burgdorferi* B31 lysate, supported by the results of von Baehr et al. [38] and Nordberg et al. [49]. von Baehr et al. tested three different *Borrelia* species in a lymphocyte transformation test and did not find any differences between these species. Nordberg et al. used *B. garinii* as a stimulating agent in an ELISpot assay among Lyme neuroborreliosis patients and obtained results which were comparable with the results of the *B. burgdorferi* B31 ELISpot assay we tested. The relatively low numbers of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 PBMCs among active Lyme neuroborreliosis patients in our study could also be explained by the compartmentalization of T cells to the CSF. Several studies have shown that patients with neurological LB had less T-cell reactivity against *Borrelia* in peripheral blood than other manifestations of LB [22, 23]. Analysis of the T-cell response in CSF and blood in a subset of patients who had neurological LB also showed a higher number of *Borrelia*-specific IFN- γ -secreting T cells in CSF than in blood [22]. Still, analysis of CSF did not result in a better diagnostic performance, as has been shown by Nordberg et al. [49], who found a sensitivity of 36% and a specificity of 82% using five spots.

In our study, we included only active Lyme neuroborreliosis patients as defined by the EFNS criteria [11]. These criteria are clear and easy to apply. Most active and treated Lyme neuroborreliosis patients in this study were deemed to have definite Lyme neuroborreliosis at the time of diagnosis (75.8% and 83.8%, respectively), and therefore, we feel confident that we were dealing with true LB cases. We used the active Lyme neuroborreliosis patients as a proxy for active LB; however, we realize that it is difficult to extrapolate the results found in this study to other manifestations of LB. Future studies should therefore include patients with other manifestations of LB as well. Our research group has started to include Lyme arthritis cases since the beginning of 2015 and intends to include other LB manifestations, such as EM, Lyme lymphocytoma, or ACA, in 2018 as well.

This study had various limitations. A difference was found in sex and age between the four study groups. Patients with active Lyme neuroborreliosis were more often male and were older than untreated healthy individuals, which is most likely explained by the way of recruitment, as most healthy individuals were recruited in our hospital, increasing the likelihood of inclusion of more (young) females. The results of the logistic regression model, however, did not show any significant attribution for sex, and the contribution of age was minimal (OR, 1.061; $P = 0.001$).

The way of recruitment also led to the inclusion of increased numbers of healthy individuals with a past tick bite and/or EM. The results of the logistic regression model indeed showed that the absence of a tick bite could aid in diagnosing active Lyme neuroborreliosis. Although most likely biased, there could be some logic in the contribution of this risk factor in developing active Lyme neuroborreliosis, because not noticing a tick bite could increase the chance of developing disseminated LB. One would expect individuals who did notice a tick bite to be more alert for

development of any symptoms suggesting LB, and those individuals would consequently seek medical advice if they developed such symptoms. They are, therefore, less likely to develop disseminated LB.

The way of recruitment of the three control groups, which included treated Lyme neuroborreliosis patients as well as healthy individuals with a previously treated early manifestation of LB and/or an increased risk of contact with the *Borrelia* bacterium, resulted in increased *Borrelia* ELISpot assay reactivity and thus lower specificity. Therefore, further studies should include cohorts with lower prevalences, as well as other (cross-reacting) diseases to better assess the specificity of the *Borrelia* ELISpot assay.

Unfortunately, not much is known about the T-cell dynamics after treatment, and controversial data have been published regarding this subject [23, 50, 51]. Therefore, this needs to be further elucidated, and we are currently monitoring the active Lyme neuroborreliosis patients both serologically and immunologically (through *Borrelia* ELISpot assay) at different time points up to 2 years after inclusion. This way we hope to get more information regarding the T-cell dynamics.

Finally, a total of six patients were included twice in this study, both as active Lyme neuroborreliosis patients and, at a later time point (≥ 1.6 years later), as treated Lyme neuroborreliosis patients. As 66.7% turned seronegative and Lyme-specific symptoms at the time of active disease had disappeared and the individuals showed either nonspecific symptoms or a complete recovery, we do not believe that this created a bias.

In conclusion, the *Borrelia* ELISpot assay used in this study, measuring the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells, cannot be used for the diagnosis of active Lyme neuroborreliosis.

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REFERENCES

1. Hofhuis A, Harms M, van den Wijngaard C, Sprong H, van PW. 2015. Continuing increase of tick bites and Lyme disease between 1994 and 2009. *Ticks Tick Borne Dis* 6:69-74. doi: 10.1016/j.ttbdis.2014.09.006.
2. Hofhuis A, Bennema S, Harms M, van Vliet AJ, Takken W, van den Wijngaard CC, Van PW. 2016. Decrease in tick bite consultations and stabilization of early Lyme borreliosis in the Netherlands in 2014 after 15 years of continuous increase. *BMC Public Health* 16:425. doi: 10.1186/s12889-016-3105-y.
3. den Boon S, Schellekens JF, Schouls LM, Suijkerbuijk AW, Docters van LB, Van PW. 2004. Doubling of the number of cases of tick bites and Lyme borreliosis seen by general practitioners in the Netherlands. [In Dutch]. *Ned Tijdschr Geneesk* 148:665-670.
4. Centers for Disease Control and Prevention (CDC). 1995. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. *MMWR Morb Mortal Wkly Rep* 44:590-1.
5. Sykes RA, Makiello P. 2016. An estimate of Lyme borreliosis incidence in Western Europe. *J Public Health (Oxf)*. doi: 10.1093/pubmed/fdw017.
6. Smith R, Takkinen J. 2006. Lyme borreliosis: Europe-wide coordinated surveillance and action needed? *Euro Surveill* 11. doi: 10.2807/esw.11.25.02977-en.
7. Gooskens J, Templeton KE, Claas EC, van Dam AP. 2006. Evaluation of an internally controlled real-time PCR targeting the *OspA* gene for detection of *Borrelia burgdorferi* sensu lato DNA in cerebrospinal fluid. *Clin Microbiol Infect* 12:894-900. doi: 10.1111/j.1469-0691.2006.01509.x.
8. Karlsson M, Hovind-Hougen K, Svenungsson B, Stiernstedt G. 1990. Cultivation and characterization of spirochetes from cerebrospinal fluid of patients with Lyme borreliosis. *J Clin Microbiol* 28:473-9. doi: 10.1128/jcm.28.3.473-479.1990.
9. Roux F, Boyer E, Jaulhac B, Dernis E, Closs-Prophette F, Puechal X. 2007. Lyme meningoradiculitis: prospective evaluation of biological diagnosis methods. *Eur J Clin Microbiol Infect Dis* 26:685-93. doi: 10.1007/s10096-007-0347-8.
10. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. 2005. Diagnosis of Lyme borreliosis. *Clin Microbiol Rev* 18:484-509. doi: 10.1128/CMR.18.3.484-509.2005.
11. Mygland A, Ljostad U, Fingerle V, Rupprecht T, Schmuthard E, Steiner I. 2010. EFNS guidelines on the diagnosis and management of European Lyme neuroborreliosis. *Eur J Neurol* 17:8-4. doi: 10.1111/j.1468-1331.2009.02862.x.
12. Leeflang MMG, Ang CW, Berkhout J, Bijlmer HA, Van BW, Brandenburg AH, van Burgel ND, van Dam AP, Dessau RB, Fingerle V, Hovius JWR, Jaulhac B, Meijer B, Van PW, Schellekens JFP, Spijker R, Stelma FF, Stanek G, Verduyn-Lunel F, Zeller H, Sprong H. 2016. The diagnostic accuracy of serological tests for Lyme borreliosis in Europe: a systematic review and meta-analysis. *BMC Infect Dis* 16. doi: 10.1186/s12879-016-1468-4.
13. Hammers-Berggren S, Hansen K, Lebech AM, Karlsson M. 1993. *Borrelia burgdorferi*-specific intrathecal antibody production in neuroborreliosis: a follow-up study. *Neurology* 43:169-75. doi: 10.1212/wnl.43.1_part_1.169.
14. Cerar T, Ogrinc K, Strle F, Ruzic-Sabljic E. 2010. Humoral immune responses in patients with Lyme neuroborreliosis. *Clin Vaccine Immunol* 17:645-50. doi: 10.1128/CVI.00341-09.
15. Stanek G, Wormser GP, Gray J, Strle F. 2012. Lyme borreliosis. *Lancet* 379:461-73. doi: 10.1016/S0140-6736(11)60103-7.
16. Nadelman RB, Luger SW, Frank E, Wisniewski M, Collins JJ, Wormser GP. 1992. Comparison of cefuroxime axetil and doxycycline in the treatment of early Lyme disease. *Ann Intern Med* 117:273-80. doi: 10.7326/0003-4819-117-4-273.
17. Wormser GP, Nadelman RB, Dattwyler RJ, Dennis DT, Shapiro ED, Steere AC, Rush TJ, Rahn DW, Coyle PK, Persing DH, Fish D, Luft BJ. 2000. Practice guidelines for the treatment of

- Lyme disease. The Infectious Diseases Society of America. Clin Infect Dis 31 Suppl 1:1-14. doi: 10.1086/314053.
18. Janský L, Reymanová P, Kopecký J. 2003. Dynamics of cytokine production in human peripheral blood mononuclear cells stimulated by LPS or infected by *Borrelia*. Physiol Res 52:593-598.
 19. Yssel H, Shanafelt MC, Soderberg C, Schneider PV, Anzola J, Peltz G. 1991. *Borrelia burgdorferi* activates a T helper type 1-like T cell subset in Lyme arthritis. J Exp Med 174:593-601. doi: 10.1084/jem.174.3.593.
 20. Gergel EI, Furie MB. 2004. Populations of human T lymphocytes that traverse the vascular endothelium stimulated by *Borrelia burgdorferi* are enriched with cells that secrete gamma interferon. Infect Immun 72:1530-1536. doi: 10.1128/IAI.72.3.1530-1536.2004.
 21. Ekerfelt C, Ernerudh J, Bunikis J, Vrethem M, Aagesen J, Roberg M, Bergstrom S, Forsberg P. 1997. Compartmentalization of antigen specific cytokine responses to the central nervous system in CNS borreliosis: secretion of IFN-gamma predominates over IL-4 secretion in response to outer surface proteins of Lyme disease *Borrelia* spirochetes. J Neuroimmunol 79:155-62. doi: 10.1016/s0165-5728(97)00118-5.
 22. Forsberg P, Ernerudh J, Ekerfelt C, Roberg M, Vrethem M, Bergstrom S. 1995. The outer surface proteins of Lyme disease *Borrelia* spirochetes stimulate T cells to secrete interferon-gamma (IFN-gamma): diagnostic and pathogenic implications. Clin Exp Immunol 101:453-60. doi: 10.1111/j.1365-2249.1995.tb03134.x.
 23. Wang WZ, Fredrikson S, Sun JB, Link H. 1995. Lyme neuroborreliosis: evidence for persistent up-regulation of *Borrelia burgdorferi*-reactive cells secreting interferon-gamma. Scand J Immunol 42:694-700. doi: 10.1111/j.1365-3083.1995.tb03713.x.
 24. Oksi J, Savolainen J, Pene J, Bousquet J, Laippala P, Viljanen MK. 1996. Decreased interleukin-4 and increased gamma interferon production by peripheral blood mononuclear cells of patients with Lyme borreliosis. Infect Immun 64:3620-3. doi: 10.1128/iai.64.9.3620-3623.1996.
 25. Gross DM, Steere AC, Huber BT. 1998. T helper 1 response is dominant and localized to the synovial fluid in patients with Lyme arthritis. J Immunol 160:1022-8.
 26. Kwaliteitsinstituut voor de Gezondheidszorg (CBO). 2013. Dutch guidelines Lyme disease 2013. Available at [In Dutch]: <https://www.rivm.nl/nieuws/cbo-richtlijn-lymeziekte-definitief>.
 27. Jin C, Roen DR, Lehmann PV, Kellermann GH. 2013. An enhanced ELISpot assay for sensitive detection of antigen-specific T cell responses to *Borrelia burgdorferi*. Cells 2:607-20. doi: 10.3390/cells2030607.
 28. Valentine-Thon E, Ilseman K, Sandkamp M. 2007. A novel lymphocyte transformation test (LTT-MELISA) for Lyme borreliosis. Diagn Microbiol Infect Dis 57:27-34. doi: 10.1016/j.diagmicrobio.2006.06.008.
 29. Brouqui P, Bacellar F, Baranton G, Birtles RJ, Bjoersdorff A, Blanco JR, Caruso G, Cinco M, Fournier PE, Francavilla E, Jensenius M, Kazar J, Laferl H, Lakos A, Lotric FS, Maurin M, Oteo JA, Parola P, Perez-Eid C, Peter O, Postic D, Raoult D, Tellez A, Tselentis Y, Wilske B. 2004. Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. Clin Microbiol Infect 10:1108-1132. doi: 10.1111/j.1469-0691.2004.01019.x.
 30. van Gorkom T, Kremer K, Voet W, Notermans DW, Vlamincx BJM, Sankatsing SUC, Thijsen SFT. 2017. Disagreement between the results from three commercial tests for the detection of *Borrelia*-specific serum antibodies in the Netherlands associated with antibiotic treatment for Lyme borreliosis: a retrospective study. Eur J Clin Microbiol Infect Dis 36:2137-2146. doi: 10.1007/s10096-017-3037-1.
 31. Liang FT, Steere AC, Marques AR, Johnson BJ, Miller JN, Philipp MT. 1999. Sensitive and specific serodiagnosis of Lyme disease by enzyme-linked immunosorbent assay with a peptide based on an immunodominant conserved region of *Borrelia burgdorferi* vlsE. J Clin

- Microbiol 37:3990-3996. doi: 10.1128/JCM.37.12.3990-3996.1999.
32. Wilske B, Fingerle V, Schulte-Spechtel U. 2007. Microbiological and serological diagnosis of Lyme borreliosis. FEMS Immunol Med Microbiol 49:13-21. doi: 10.1111/j.1574-695X.2006.00139.x.
 33. Hansen K, Lebech AM. 1991. Lyme neuroborreliosis: a new sensitive diagnostic assay for intrathecal synthesis of *Borrelia burgdorferi*-specific immunoglobulin G, A, and M. Ann Neurol 30:197-205. doi: 10.1002/ana.410300212.
 34. Bouwman JJM, Thijsen SFT, Bossink AW. 2012. Improving the timeframe between blood collection and interferon gamma release assay using T-Cell Xtend. J Infect 64:197-203. doi: 10.1016/j.jinf.2011.10.017.
 35. Feske ML, Medina M, Graviss EA, Lewis DE. 2012. IL-7 addition increases spot size and number as measured by T-SPOT.TB. Methods Mol Biol 792:229-41. doi: 10.1007/978-1-61779-325-7_18.
 36. Dattwyler RJ, Thomas JA, Benach JL, Golightly MG. 1986. Cellular immune response in Lyme disease: the response to mitogens, live *Borrelia burgdorferi*, NK cell function and lymphocyte subsets. Zentralbl Bakteriol Mikrobiol Hyg A 263:151-9. doi: 10.1016/S0176-6724(86)80118-3.
 37. Dattwyler RJ, Volkman DJ, Halperin JJ, Luft BJ, Thomas J, Golightly MG. 1988. Specific immune responses in Lyme borreliosis. Characterization of T cell and B cell responses to *Borrelia burgdorferi*. Ann N Y Acad Sci 539:93-102. doi: 10.1111/j.1749-6632.1988.tb31842.x.
 38. von Baehr V, Doebis C, Volk HD, von Baehr R. 2012. The lymphocyte transformation test for *Borrelia* detects active Lyme borreliosis and verifies effective antibiotic treatment. Open Neurol J 6:104-12. doi: 10.2174/1874205X01206010104.
 39. Ljostad U, Mygland A. 2010. Remaining complaints 1 year after treatment for acute Lyme neuroborreliosis; frequency, pattern and risk factors. Eur J Neurol 17:118-123. doi: 10.1111/j.1468-1331.2009.02756.x.
 40. Vrethem M, Hellblom L, Widlund M, Ahl M, Danielsson O, Ernerudh J, Forsberg P. 2002. Chronic symptoms are common in patients with neuroborreliosis - a questionnaire follow-up study. Acta Neurol Scand 106:205-8. doi: 10.1034/j.1600-0404.2002.01358.x.
 41. Treib J, Fernandez A, Haass A, Grauer MT, Holzer G, Woessner R. 1998. Clinical and serologic follow-up in patients with neuroborreliosis. Neurology 51:1489-91. doi: 10.1212/wnl.51.5.1489.
 42. Ekerfelt C, Forsberg P, Svenvik M, Roberg M, Bergstrom S, Ernerudh J. 1999. Asymptomatic *Borrelia*-seropositive individuals display the same incidence of *Borrelia*-specific interferon-gamma (IFN-gamma)-secreting cells in blood as patients with clinical *Borrelia* infection. Clin Exp Immunol 115:498-502. doi: 10.1046/j.1365-2249.1999.00840.x.
 43. Dattwyler RJ, Volkman DJ, Luft BJ, Halperin JJ, Thomas J, Golightly MG. 1988. Seronegative Lyme disease. Dissociation of specific T- and B-lymphocyte responses to *Borrelia burgdorferi*. N Engl J Med 319:1441-1446. doi: 10.1056/NEJM198812013192203.
 44. Kanevskiy LM, Telford WG, Sapozhnikov AM, Kovalenko EI. 2013. Lipopolysaccharide induces IFN-gamma production in human NK cells. Front Immunol 4:11. doi: 10.3389/fimmu.2013.00011.
 45. Varma TK, Lin CY, Toliver-Kinsky TE, Sherwood ER. 2002. Endotoxin-induced gamma interferon production: contributing cell types and key regulatory factors. Clin Diagn Lab Immunol 9:530-43. doi: 10.1128/cdli.9.3.530-543.2002.
 46. Lehmann PV. 2005. Image analysis and data management of ELISPOT assay results. Methods Mol Biol 302:117-32. doi: 10.1385/1-59259-903-6:117.
 47. Coipan EC, Jahfari S, Fonville M, Oei GA, Spanjaard L, Takumi K, Hovius JW, Sprong H. 2016. Imbalanced presence of *Borrelia burgdorferi* s.l. multilocus sequence types in clinical manifestations of Lyme borreliosis. Infect Genet Evol 42:66-76. doi: 10.1016/j.

- meegid.2016.04.019.
48. Strle F, Ruzic-Sabljic E, Cimperman J, Lotric-Furlan S, Maraspin V. 2006. Comparison of findings for patients with *Borrelia garinii* and *Borrelia afzelii* isolated from cerebrospinal fluid. Clin Infect Dis 43:704-10. doi: 10.1086/506936.
 49. Nordberg M, Forsberg P, Nyman D, Skogman BH, Nyberg C, Ernerudh J, Eliasson I, Ekerfelt C. 2012. Can ELISpot be applied to a clinical setting as a diagnostic utility for neuroborreliosis? Cells 1:153-67. doi: 10.3390/cells1020153.
 50. Zoschke DC, Skemp AA, Defosse DL. 1991. Lymphoproliferative responses to *Borrelia burgdorferi* in Lyme disease. Ann Intern Med 114:285-9. doi: 10.7326/0003-4819-114-4-285.
 51. Callister SM, Jobe DA, Stuparic-Stancic A, Miyamasu M, Boyle J, Dattwyler RJ, Arnaboldi PM. 2016. Detection of IFN-gamma secretion by T cells collected before and after successful treatment of early Lyme disease. Clin Infect Dis 62:1235-1241. doi: 10.1093/cid/ciw112.

SUPPLEMENTAL MATERIAL

Table S1. Antibody index and *Borrelia* ELISpot assay results among the active Lyme neuroborreliosis patients in this study

Test ^a	Active Lyme neuroborreliosis patients (<i>n</i> = 33)		
	NEG	POS	<i>P</i> value
Ig total AI result ^b (<i>n</i> ; %)	8 (24.2)	25 (75.8)	
Median <i>Borrelia</i> ELISpot result ^c (IQR)	8.0 (2.3-21.8)	6.0 (0.0-10.8)	0.550
IgM AI result (<i>n</i> ; %)	17 (51.5)	16 (48.5)	
Median AI score ^d (IQR)	<0.3	3.9 (3.2-15.6) ^e	
Median <i>Borrelia</i> ELISpot result ^c (IQR)	2.0 (0.0-14.0)	6.8 (5.0-15.8)	0.081
IgG AI result (<i>n</i> ; %)	11 (33.3)	22 (66.7)	
Median AI score ^d (IQR)	<0.3	11.1 (2.9-27.1)	
Median <i>Borrelia</i> ELISpot result ^c (IQR)	6.0 (3.0-24.0)	5.5 (0.0-10.1)	0.336

NEG, negative; POS, positive

a. Ig: immunoglobulin; AI: antibody index; IQR: interquartile range.

b. The Ig total AI result is based on a combination of the results of both immunoglobulins: negative when both the IgM AI result and the IgG AI result are negative, and positive when at least one of these is positive.

c. The *Borrelia* ELISpot result is based on the numbers of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 peripheral blood mononuclear cells among all active Lyme neuroborreliosis patients.

d. An AI score ≥ 0.3 is regarded as positive.

e. For four active Lyme neuroborreliosis patients, the IgM AI scores were lacking; however, in the electronic patient files the final IgM result for all four were recorded as positive.

Table S2. Various logistic regression models assessing risk factors which could contribute to the diagnostic performance of the *Borrelia* ELISpot assay used in this study. The logistic regression model calculates the added value of various risk factors for determining active Lyme neuroborreliosis; only a few (combinations of) risk factors are shown.

Covariates logistic regression ^a	Logistic regression models				
	1 (n = 243)	2 (n = 234 ^d)	3 (n = 234 ^d)	4 (n = 234 ^d)	5 ^e (n = 234 ^d)
Factors (P values)					
Gender (male)				0.071	0.106
Tick bite (no)		0.055	0.054	0.037	0.029
EM (no)				0.964	0.593
Continuous variables (P values)					
Age		0.012	0.013	0.014	0.001
<i>Borrelia</i> ELISpot result ^b	0.126		0.841	0.872	0.010
Interaction term (P values)					
Age by <i>Borrelia</i> ELISpot ^b					0.018
AUC	0.591	0.689	0.694	0.741	0.769
Model fit ^c	0.026	0.733	0.726	0.519	0.809
Odds ratio (95% CI for OR)					
Gender (male)				2.282 (0.931-5.595)	2.117 (0.853-5.256)
Tick bite (no)		2.334 (0.981-5.555)	2.351 (0.985-5.612)	2.690 (1.062-6.815)	2.938 (1.119-7.710)
EM (no)				0.972 (0.281-3.365)	0.706 (0.197-2.529)
Age		1.039 (1.008-1.071)	1.039 (1.008-1.071)	1.038 (1.007-1.069)	1.061 (1.024-1.101)
<i>Borrelia</i> ELISpot result ^b	1.016 (0.995-1.037)		1.003 (0.975-1.032)	1.002 (0.974-1.031)	1.218 (1.049-1.415)
Age by <i>Borrelia</i> ELISpot result ^b					0.996 (0.993-0.999)

a. EM, erythema migrans; AUC, area under the curve; CI, confidence interval; OR, odds ratio.

b. The *Borrelia* ELISpot result is based on the number of *B. burgdorferi* B31-specific IFN- γ -secreting T cells/ 2.5×10^5 peripheral blood mononuclear cells.

c. The Hosmer-Lemeshow goodness of fit test was used to assess whether the model fitted the data.

d. Nine active Lyme neuroborreliosis patients did not complete the Lyme-specific questionnaire, so data on tick bite and erythema migrans was not available. Therefore, models including one or both of these risk factors were based on a total of 234 cases instead of 243.

e. The randomForest method using the Gini coefficient (R studio, version 1.1.383, 2009-2017 RStudio, Inc.) was also performed and confirmed that model 5 fitted the data best (data not shown).