Immunodiagnostics of Lyme neuroborreliosis

Gorkom, T. van

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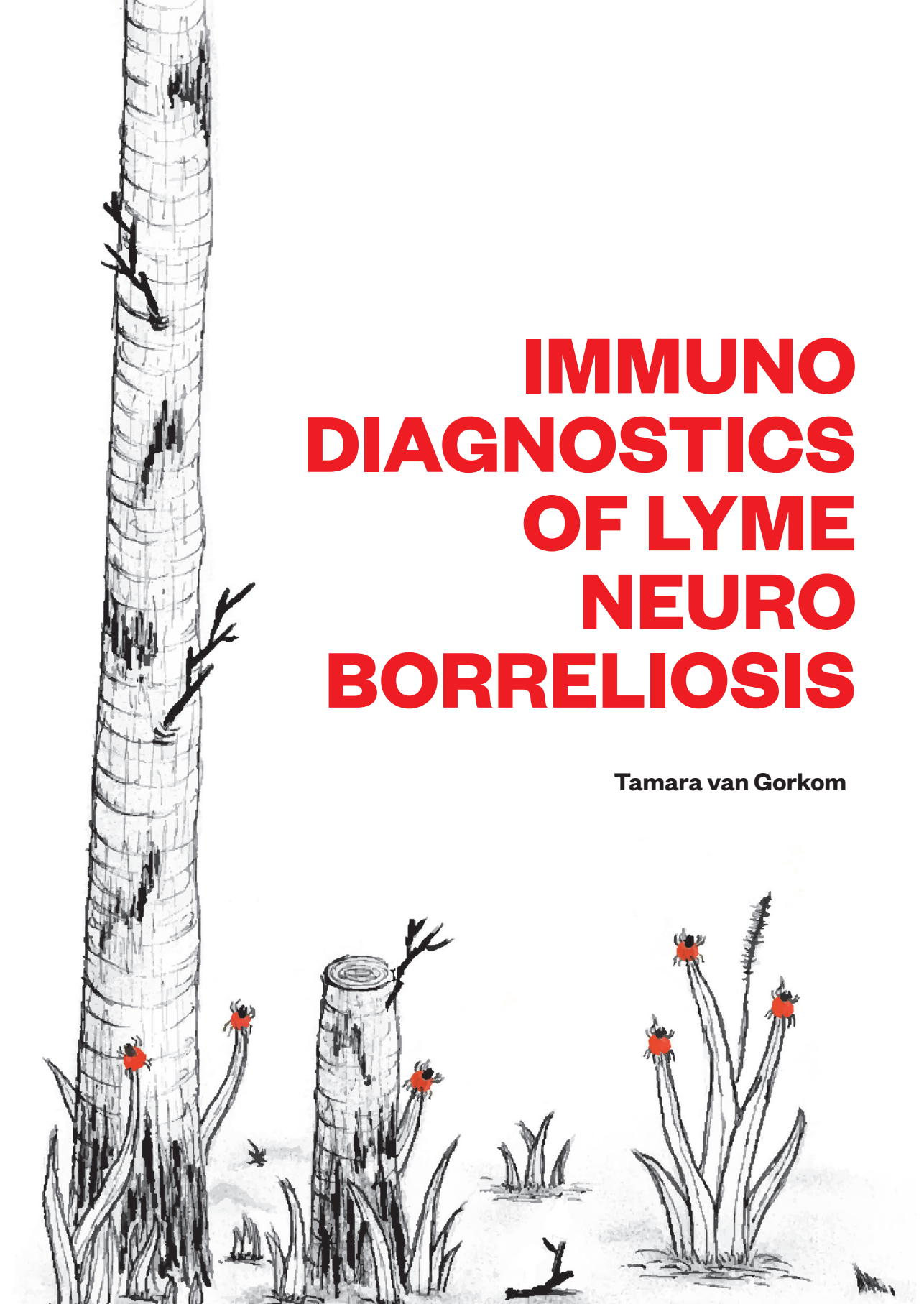
Tamara van Gorkom



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TAMARA VAN GORKOM

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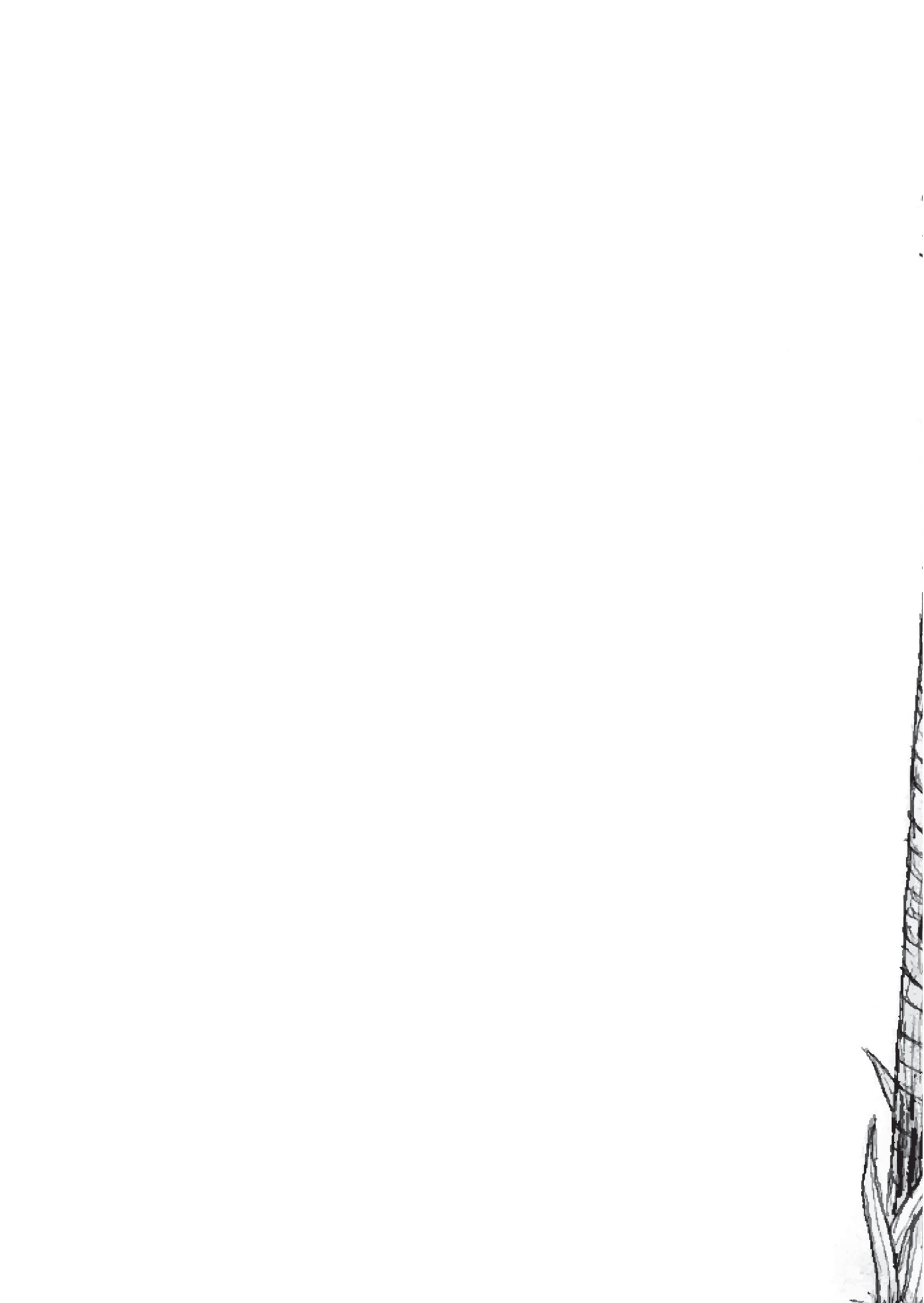

1 GENERAL INTRODUCTION

INTRODUCTION

Lyme disease, also known as Lyme borreliosis (LB), is the most common tick-borne disease in temperate regions of the Northern Hemisphere [1]. LB is a multisystem disease, and the most frequent clinical symptom is an expanding skin rash also known as erythema migrans (EM). Other manifestations can involve the peripheral and/or central nervous system (Lyme neuroborreliosis [LNB]), joints (Lyme arthritis [LA]), skin (acrodermatitis chronica atrophicans [ACA]), and rarely the heart (Lyme carditis) and eyes (ocular Lyme) [1]. Except for a typical EM rash, the diagnosis of most other manifestations can be a challenge as symptoms are often non-pathognomonic [2]. This introduction provides background on the epidemiology, pathology and diagnostic challenges of LB. At the end of the chapter, the aims of this thesis are outlined.

THE HISTORY OF LYME BORRELIOSIS

The history of LB covers more than one century. The disease is named after the place Old Lyme (Connecticut, USA) where a clustered outbreak of presumed juvenile rheumatoid arthritis took place in the period between 1972 and 1975 [3]. Approximately one quarter of the cases reported an expanding skin rash currently known as EM in the weeks preceding the arthritis. In retrospect, this skin manifestation had already been described by Afzelius in 1910 [4] and by Lipschutz in 1913 [5] and was linked to the bite of a tick [6]. As the disease in Old Lyme did not match other known causes of arthritis it was considered to represent a new disease and was consequently called 'Lyme arthritis' [3]. In 1922, the first report of an association between EM and nervous system complaints appeared when Garin and Bujadoux reported a painful meningoradiculitis after the bite of a tick [7]. This was followed by a report from Hellerström in 1930 [8], who linked EM with meningitis. Nervous system complaints were also described by the German neurologist Bannwarth in 1941 and 1944 [9, 10]. He described a manifestation, now known as Bannwarth's syndrome, which was characterized by intense nerve pains that radiated to the extremities. Often patients also showed peripheral nervous system involvement (mainly facial nerve palsy) with an elevated number of lymphocytes in the cerebrospinal fluid (CSF).

A few years later, in 1948, Lennhoff [11] developed a staining technique that showed spirochetes in the lesions of patients with EM; however, these findings were not confirmed until 1981 when Burgdorfer et al. [12] discovered a new spiral-shaped bacterium (so-called spirochetes) in an *Ixodes dammini* (now: *Ixodes scapularis*) tick and called it *Borrelia burgdorferi.* He also suggested that this spirochete might be associated with Lyme disease as antibodies from Lyme disease patients bound to the bacterium. This association was confirmed in 1983 by Steere et al. [13], who showed that spirochetes that were isolated from ticks and Lyme disease patients had similar morphological and immunological features. They also showed increased levels of Lyme spirochete-specific immunoglobulin (Ig)M and IgG in Lyme disease patients that were absent in controls. Around the same time, Stiernstedt et al. [14] described 35 patients in Sweden who suffered from chronic meningitis, which was sometimes preceded by an EM or tick bite. Most of these patients had antibodies against the Lyme spirochete as well as against the *Ixodes ricinus* tick and, thus, an association between these spirochetes, a tick bite and chronic meningitis was demonstrated in Europe as well. Since then, numerous reports have been published on LB, which is now considered the most prevalent tick-borne infection with a wide geographic distribution in Northern America, Europe and parts of Asia [15].

THE VECTORS AND PATHOGENS OF LYME BORRELIOSIS

The genus *Borrelia* consists of two major phylogenetic groups: (i) the relapsing fever *Borrelia* group and (ii) the *B. burgdorferi* sensu lato (s.l.) complex group [16]. The genospecies which are part of the *B. burgdorferi* s.l. complex have been linked to LB and comprises at least 20 genospecies [16]. In North America, the most important genospecies causing LB is *B. burgdorferi* sensu stricto (s.s.) [16]. In Europe, the predominant genospecies causing LB are *Borrelia afzelii, Borrelia garinii, Borrelia bavariensis* and *B. burgdorferi* s.s. [16].

LB is a zoonosis and *B. burgdorferi* s.l. is transmitted by hard-bodied ticks of the *Ixodes* complex [17, 18]. The occurrence of LB in the world is dependent on the geographical distribution of its vector and reservoir hosts and is, therefore, mainly found in the Northern Hemisphere [19, 20]. In North America, the main tick species are *I. scapularis* in the Northeast and Upper Midwest and *Ixodes pacificus* in the West (Figure 1) [21]. In Europe, the main vector is *I. ricinus* and in Asia *Ixodes persulcatus*, although *I. persulcatus* has also been found in Eastern Europe [21].

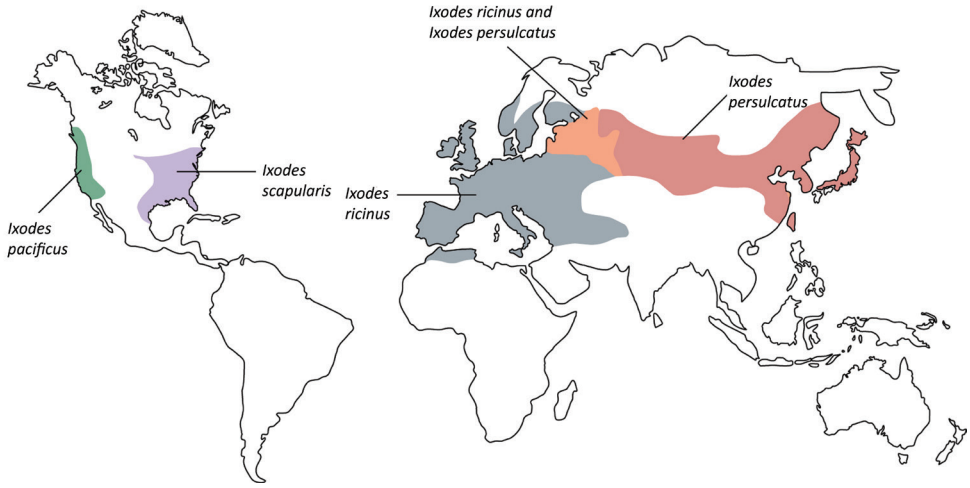

Fig. 1. Global distribution of the hard-bodied ticks of the *Ixodes* complex that can transmit *B. burgdorferi* s.l.. Figure reprinted from The Lancet, 379(9814), Stanek G, Wormser GP, Gray J, Strle F. Lyme borreliosis, 461-73, ©2012 [21], with permission from Elsevier.

Ixodes ticks have a life cycle from 2 to 6 years that consists of four life stages: egg, larva, nymph and adult stage (Figure 2) [21]. Most tick activity is seen in spring and summer, which explains the seasonal incidence of LB as most cases occur between May and November [22, 23].

THE PREVALENCE OF *BORRELIA* GENOSPECIES IN TICKS IN THE NETHERLANDS

In the Netherlands, a study investigating questing *I. ricinus* ticks (*n* = 5570) from 22 different study sites showed a prevalence of *B. burgdorferi* s.l. of 11.8%, which could be subdivided in *B. afzelii* (6.7%), *B. garinii/B. bavariensis* (1.5%), *Borrelia valaisiana* (1.2%), *B. burgdorferi* s.s. (0.2%), and non-typeable *Borrelia* (2.2%) [24]. Another study investigated the prevalence of *B. burgdorferi* s.l. in 314 ticks that were obtained from 293 patients [25] in the Netherlands. The majority (94%) of these ticks were *I. ricinus* ticks, and almost one third (29.3%) contained *B. burgdorferi* s.l. DNA, which could be subdivided in *B. afzelii* (11.5%), *B. garinii* (3.5%), *B. burgdorferi* s.s. (2.2%), *B. valaisiana* (1.3%), and non-typeable *Borrelia* (11.5%) [25].

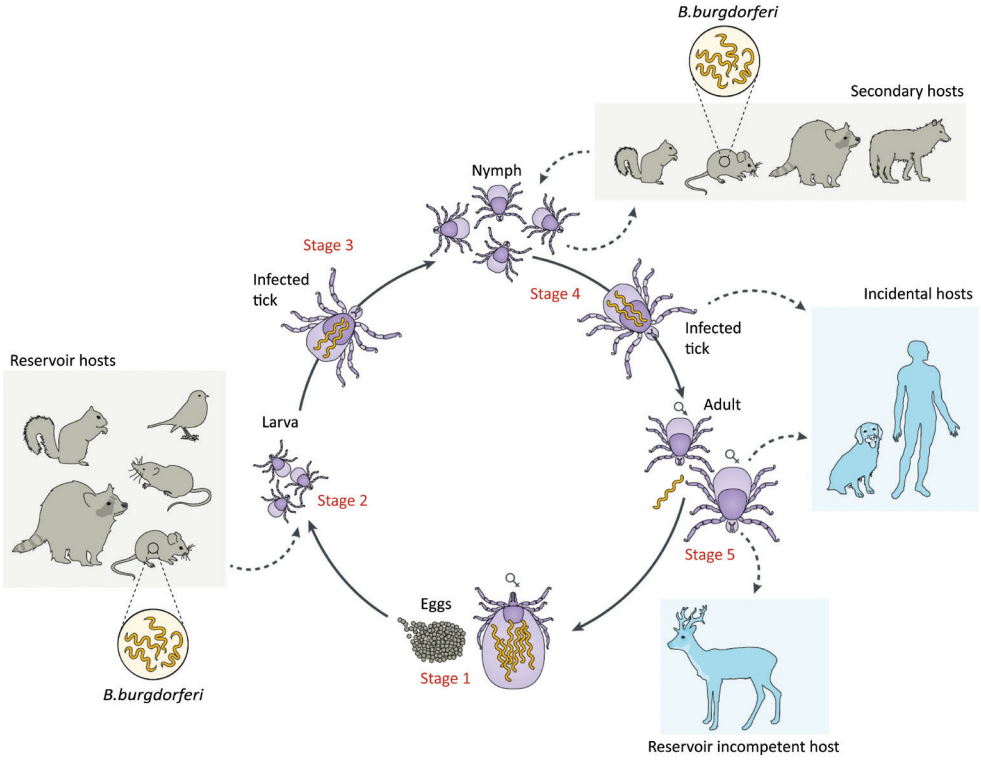

Fig. 2. The life cycle of the *Ixodes* tick and *B. burgdorferi* s.l.. The adult female tick lays up to 2000 eggs (stage 1), which will develop into six-legged larva (stage 2) [21, 26]. A blood meal is needed for development into the nymphal stage and this is probably the first moment the ticks may become infected with *B. burgdorferi* s.l. as transovarial transmission does not seem to occur [27, 28]. Larva typically feed on small mammals and birds and a blood meal normally takes 3-7 days. After the blood meal, the larva will drop off and molt into an eight-legged nymph (stage 3). Nymphs typically feed on medium to large-sized mammals and this is a second moment the tick may become infected with *B. burgdorferi* s.l.. After the blood meal, nymphs will drop off and molt into the adult stage (stage 4). Adult ticks typically seek a large animal host, such as deer, for mating and a last blood meal (stage 5). Since ticks cannot acquire *B. burgdorferi* s.l. from deer, they are called reservoir-incompetent hosts [26, 29]. Deer are, however, important for maintaining the tick population. For all feeding stages, humans can be an incidental host [26]. Figure adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Microbiology. Interactions between *Borrelia burgdorferi* and ticks. Kurokawa C, Lynn GE, Pedra JHF, Pal U, Narasimhan S, Fikrig E, ©2020 [26].

THE EPIDEMIOLOGY OF LYME BORRELIOSIS

LB has a bimodal age distribution with an incidence peak in childhood (range between 5 and 15 years) and one in adulthood (range between 50 to 75 years) [20, 30-35]. In North America, LB is a notifiable disease since 1991 and about 30,000 confirmed and probable LB cases are reported to the Centers of Disease Control (CDC) each year [35, 36]. Reported LB cases include dermatologic, rheumatologic, neurologic, and cardiac manifestations; the majority (70%-80%) are EM cases [35, 36]. As the number of reported LB cases is based on passive reporting, the reported incidence could be an underestimation, and recent CDC estimates suggest that the actual number of LB cases might be 10 times higher [36]. In Asia, reports on the incidence rates of LB are limited, although LB cases have been reported in Korea and Japan, where LB is a notifiable disease [37], and also in Russia [38], China [39] and Mongolia [40]. In most European countries, LB is not notifiable and to gain insight into the LB incidence rates, different strategies are used.

For the most part, incidence rates of LB in Europe are based on positive test results obtained from diagnostic laboratories [41]. Other ways to obtain LB incidence rates are based on voluntary reporting, surveys conducted among physicians, or by hospital diagnoses [41]. It is estimated that LB affects around 85,000 patients in Europe each year [20], although this is also most likely an underestimation. The incidence rate of LB in Western Europe was estimated at 22 per 100,000 population per year [42]. The highest incidences rates were found in Norway [32], Sweden [43], Switzerland [44], Austria [20], Slovenia [45] and the Netherlands [46], where the incidence rates ranged between 100 and 500 per 100,000 population per year. Incidence rates between 25 and 100 per 100,000 population per year were found in Finland [47], Germany [34], Czech Republic [48], France [49], Belgium [50] and Lithuania [51]. The lowest incidence rates, of less than 10 per 100,000 population per year, were found in the United Kingdom [52], Italy [53], Spain [54] and Iceland [55].

Reported incidence rates, however, should be interpreted with caution as these sometimes reflect only certain regions of a country or can be based on the mean incidence rate of the whole country. Use of non-standardized case definitions [42, 56] and unclear symptomology reporting [57] can also result in biased incidence rates. For EM, which is a clinical diagnosis, data might be lacking as not all LB cases will develop an EM or an EM remains unnoticed or unrecognized [42, 58, 59]. When serology is part of the case definition, this can also result in biased estimates: e.g., under-reporting due to false-negative test results can occur in the first weeks of infection when antibody levels are rising, but are still undetectable at the moment of blood sampling [42]. On the other hand, over-reporting can occur when positive serology results are interpreted as proof of active disease, while these can also result from a past infection [57]. Despite these pitfalls, in both North America [60] and Europe [56, 61], the incidence of LB seems to increase. Factors contributing to this increase include climate change, expansion of ticks and reservoir hosts, increased human tick exposure, more awareness as well as improved monitoring, detection, and reporting of both ticks and LB diagnoses [20, 62].

THE EPIDEMIOLOGY OF LYME BORRELIOSIS IN THE NETHERLANDS

In the period between 1994 and 2009, the incidence of tick bite consultations and EM diagnoses in the general practice in the Netherlands was investigated and both showed a 3-fold increase (Figure 3) [63-66]. This rise is likely due to, at least in part, an increase in disease awareness. In 2014, the number of tick bite consultations in the Netherlands slightly decreased and the number of EM cases seemed to stabilize [67], and in 2017, the number of tick bite consultations and EM diagnoses increased again [46].

The risk of transmission of *B. burgdorferi* s.l. among patients that visited the general practitioner (GP) for tick bites or EM in the Netherlands has been assessed in a nationwide study conducted in 2015 [68]. The findings indicate that the risk of developing EM after a tick bite was 2.6%, and synthesis *of Borrelia*-specific antibodies was shown in 3.2% of the tick bite cases. Analysis among patients who were bitten by a *B. burgdorferi* s.l.-positive tick showed an increased risk of developing EM as well as an increased risk of seroconversion (4.4% and 5.9%, respectively). Another Dutch study showed a similar risk of 2.6% for developing LB after a tick bite and showed that this was positively associated with tick attachment time, tick engorgement and the presence of *B. burgdorferi* s.l. DNA in ticks. The highest risk for developing LB was 14.4% and was linked to a substantially engorged *B. burgdorferi* s.l.-positive tick [69]. In Europe, transmission of *B. burgdorferi* s.l. by adult ticks is said to occur after at least 24 hours of tick infestation. However, in mouse models transmission was seen within 12 hours by nymphal ticks and this shorter infection time could be explained by *B. burgdorferi* s.l. already present in the salivary glands before a blood meal was initiated [70].

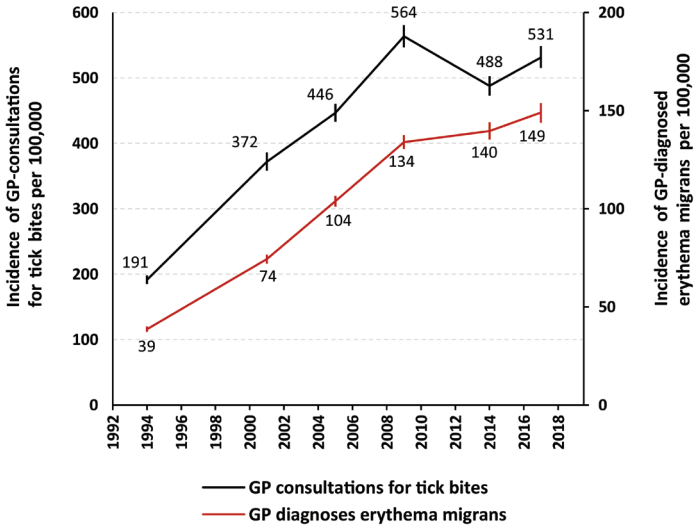

Fig. 3. The incidence of general practitioner (GP) reported tick bite consultations and erythema migrans (EM) diagnoses in the general practice between 1994 and 2017 in the Netherlands (17.1 million pop. in 2017). Figure adapted by permission from van den Wijngaard et al. [46].

THE CLINICAL MANIFESTATIONS OF LYME BORRELIOSIS

LB is a multisystem disease that can involve skin, nervous system, joints, heart, and eyes and is often classified as an early localized, early disseminated, or late disseminated disease (Figure 4A) [2]. Dissemination of *B. burgdorferi* s.l. can occur by flagellum-induced motility and chemotaxis [71, 72], and might, in part, be dependent on the genospecies causing LB. In North America, dissemination via blood seems more likely given the higher rates of spirochetes in the blood of patients from North America [73, 74]. In Europe, dissemination via peripheral nerves seems more likely, at least among patients with meningoradiculitis, which is most often caused by *B. garinii* [75, 76]. Patients with meningoradiculitis also have a higher frequency of EM located on the head, neck or torso compared to EM patients without neurological involvement and for the majority of these cases (79%), the location of the EM matched the location of the radicular pain [77].

The different Lyme manifestations (Figure 4B) might, in part, be explained by the different genospecies, which are linked to different tissue tropisms: e.g., *B. afzelii* is associated with skin manifestations, *B. burgdorferi* s.s. with joint manifestations, and *B. garinii* and *B. bavariensis* with nervous system manifestations [15, 16, 75, 78, 79]. The difference in clinical presentation might also be explained by the different genospecies as the EM of patients in North America, which is caused by *B. burgdorferi* s.s., expands more rapidly and is more often accompanied with other symptoms than EM in European patients, which is mainly caused by *B. afzelii* or *B. garinii* [73, 80]. Intra-species variation and host genetic factors have also been linked to differences in disease severity. For instance, in North America, *B. burgdorferi* s.s. outer surface protein (Osp)C type A in LA patients with a polymorphism in toll-like receptor 1 is associated with more inflammation and more severe LA [81, 82]. In Northeastern America, 65% of 291 strains isolated from EM lesions were attributable to four *B. burgdorferi* s.s. OspC types (A, B, I and K), and in the upper Midwest, *B. burgdorferi* s.s. OspC type H was mostly found (18.5% of 65 strains) [83]. In Europe, only certain *B. burgdorferi* s.l. sequence types (based on eight housekeeping genes) are found to cause LB in humans [79].

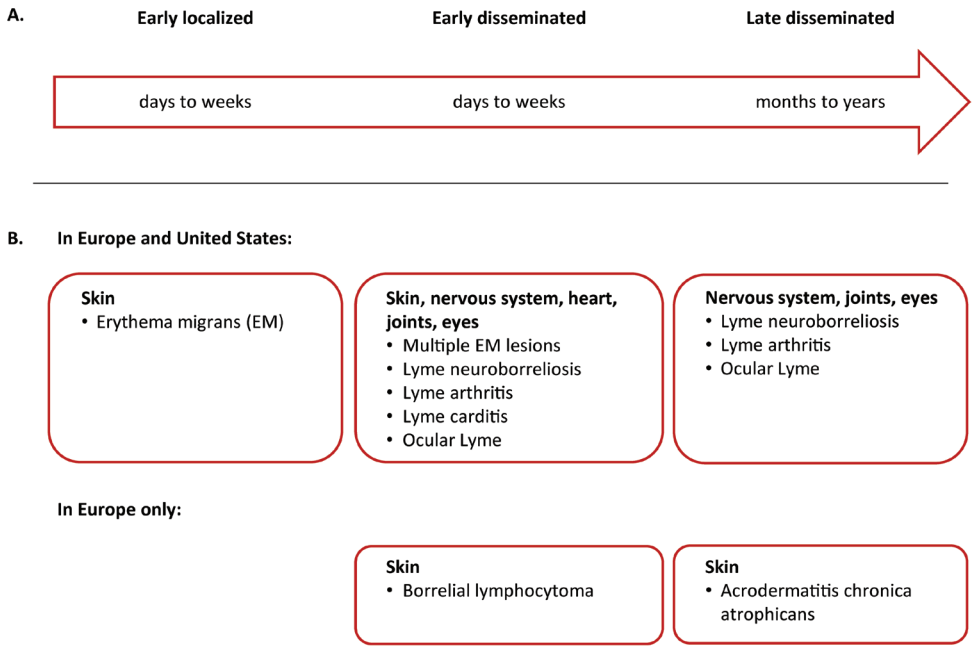

Fig. 4. The classification (panel A) and manifestations (panel B) of Lyme borreliosis seen in Europe and North America. Figure adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Reviews Disease Primers. Lyme borreliosis. Steere AC, Strle F, Wormser GP, Hu LT, Branda JA, Hovius JW, Li X, Mead PS, ©2016 [80].

Asymptomatic infections occur as well and in Europe, these seem to be as common as symptomatic infections [25, 84]. In North America, however, asymptomatic infections are less common than symptomatic infections as was shown by Steere et al. [85], who reported that clinical symptoms were absent in less than 7% of the participants who showed seroconversion to *B. burgdorferi*.

ERYTHEMA MIGRANS

EM is the most prevalent LB manifestation and occurs in about 70% to 95% [2, 31, 34, 36, 50] of all LB cases. It is the only manifestation of early localized LB and occurs several days to weeks (typically 7 to 14 days) after the tick bite, when a skin rash appears that expands over time [16, 86]. This expanding skin rash is caused by the centrifugal migration through the skin of *B. burgdorferi* s.l. from the site of the tick bite [87]. A skin lesion that is equal to or more than 5 cm in diameter is often needed for a clinical diagnosis [21, 88]. Smaller lesions may be considered as EM when at least 2 days have past and the lesion is expanding. The most typical EM feature is a bull's-eye rash, but central clearing in EM lesions may not always occur (Figure 5) [21]. Other symptoms that can occur in approximately 50% of the patients are itching, burning or pain at the site of the EM, and, less often, fatigue, headache, malaise, arthralgias and myalgias [2]. In most areas of Europe, EM is caused by *B. afzelii* (70% to 90%), and less frequently by *B. garinii* (10% to 20%), except in Northeastern Europe where *B. garinii* predominates [2]. In North America, EM patients more often experience fever and lymphadenopathy than European EM patients [2, 89], and central clearing of EM is more often seen in Europe [16, 89]. If an EM remains unnoticed or unrecognized and left untreated, *B. burgdorferi* s.l. can spread to other body parts, and cause (early) disseminated LB.

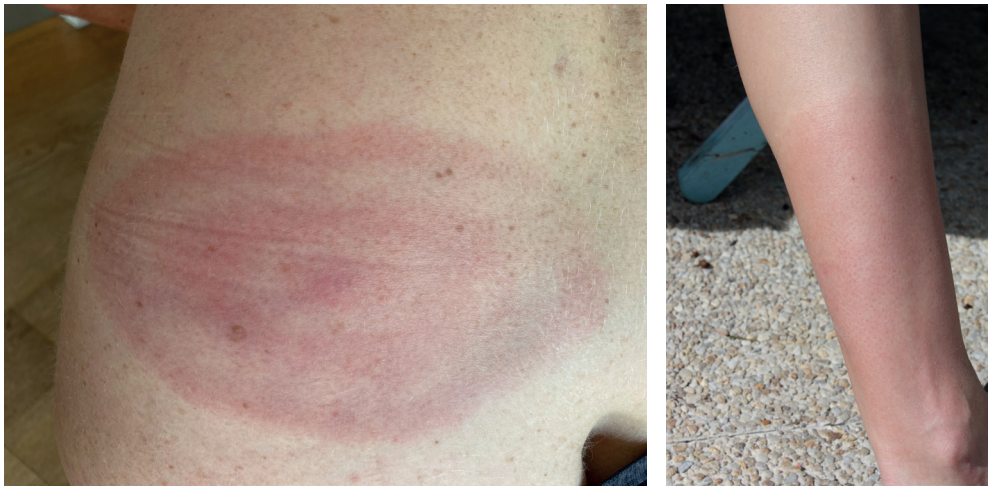

Fig. 5. A classical bull's-eye rash (left panel) and a non-typical EM lesion (right panel). Photos from private collection.

MULTIPLE ERYTHEMA MIGRANS

The occurrence of multiple EM lesions at the time of presentation is found in 4% to 50% of the EM patients in Europe [86, 90] and in 20% to 40% of the EM patients in North America [91, 92]. In general, multiple EM lesions are smaller than single EM lesions and often lack the indurated center seen in single EM lesions [2]. The number of EM lesions in European patients often ranges between two to six lesions, which is lower than the number of EM lesions seen in patients from North America (>20 lesions) [93].

BORRELIAL LYMPHOCYTOMA

Borrelial lymphocytoma is mainly observed in Europe and occurs in less than 8% of LB cases [31, 44]. It is often caused by *B. afzelii* and, consistent with the geographical spread of the bacterium, not seen in North America [89]. Borrelial lymphocytoma appears as a painless small swelling of the skin that slowly enlarges to a diameter of up to several centimeters and is seen more often in children (1.5% to 7%) than in adults (0.5% to 2%) and is mostly located on the earlobe or areola mammae [2].

LYME CARDITIS

Heart involvement leading to Lyme carditis occurs in 0.3% to 4% of LB cases in Europe and in approximately 1% of LB cases in the U.S. [94], and mostly starts within 2 months after the onset of infection [2]. Lyme carditis most commonly involves conduction system disturbances of the atrioventricular (AV) node resulting in various degrees of AV block, but other beat disturbances, endomyocarditis and pericarditis have also been reported [1]. It is often preceded by an EM and can include symptoms of the nervous system or joints [1].

OCULAR LYME

Ocular manifestations have been reported during the early and later stages of disseminated LB. In the first few weeks, conjunctivitis has been reported in up to 10% of patients, and keratitis, uveitis and vitritis have also been reported [95]. Involvement of the eyes can also occur in conjunction with other Lyme manifestations, such as LA [96], and cause symptoms such as keratitis or uveitis. In patients with LNB, palpebral diastasis, blurred vision, strabismus and diplopia can occur [97].

LYME ARTHRITIS

LA is more commonly found among LB cases in North America (25%) [98] than in Europe (2% to 7%) [31, 34, 44]. Most patients have intermittent or persistent attacks of swelling and pain in one joint, most often the knee, although other large or small joints may also be affected, such as the ankle, hip, shoulder, elbow, or wrist [99, 100]. LA is seen as an early and late Lyme manifestation and in North America, and the onset of disease can range from 4 days to 2 years, with a mean of 6 months. In Europe, the onset of disease can range from 10 days to 16 months, with a mean of 3 months [2].

ACRODERMATITIS CHRONICA ATROPHICANS

ACA is a chronic skin manifestation, which occurs in 1% to 6% of European LB cases [31, 44, 101]. It is observed more often among women and rarely occurs among children [2]. ACA is mostly caused by *B. afzelii,* although infections caused by *B. garinii* and *B. burgdorferi* s.s. are also observed [2]. The affected skin mostly comprises the distal parts of the extremities and is characterized by a red or bluish-red discoloration with or without swelling that can become atrophic over time [2]. In contrast to EM and borrelial lymphocytoma, ACA is not self-limiting, and a substantial number of ACA patients develop a sensory peripheral neuropathy when left untreated [2].

THE CLINICAL MANIFESTATIONS OF LYME BORRELIOSIS IN THE NETHERLANDS

In 2010, the incidence rate of EM and disseminated LB was assessed by conducting a nationwide survey among GPs, company physicians, and medical specialists involved in LB diagnosis [68]. Based on the results of this study, the estimated number of EM cases was 22,000 and the estimated number of disseminated LB cases was 1,300. The majority of disseminated LB cases either had LNB or LA (34% and 39%, respectively). Other reported disseminated Lyme manifestations included ACA, borrelial lymphocytoma, Lyme carditis and ocular Lyme (16%, 8%, 1% and 1%, respectively). Based on the incidence rates of all LB cases in 2010, the relative proportion of patients with EM was 91%, with LA 2.1%, with LNB 1.8%, and with ACA 0.8% [67, 68]. A similar GP survey carried out in 2017 showed that the estimated number of EM cases had increased to 25,500, and that of disseminated LB to 1,500 cases nationwide (cf. Figure 3) [46].

THE CLINICAL MANIFESTATIONS OF LYME NEUROBORRELIOSIS

LNB is seen in <1% to 16% of LB cases in Europe [2, 31, 34, 44, 50, 102] and in approximately 12.5% of confirmed LB cases in North America [35]. Most LNB patients (90% to 95%) are classified as early LNB (symptom duration of less than 6 months) [103-105]. In Europe, less than 60% of the LNB patients noticed a tick bite and/or EM [76, 106-109]. For patients with early LNB, both the peripheral nervous system as well as the central nervous system can be affected. In Europe, the most common manifestation of LNB among adults is a lymphocytic meningoradiculitis (Bannwarth's syndrome), which presents as a severe sharp neuropathic pain of the skin that intensifies at night [76, 107, 109]. The radicular pain is often located at the site of the tick bite or EM [77], and is the only symptom present in almost half of the patients [107]. Approximately 40% of the patients have cranial nerve involvement comprising the facial nerve (nerve VII), that can result in either a unilateral or a bilateral (<30% of cases) paresis [76, 105, 107, 109]. Other cranial nerves that are less frequently involved include the abducens nerve (nerve VI), and the oculomotor nerve (nerve III), and rarely other cranial nerves [105, 107, 109, 110]. Limb, phrenic, abdominal wall, and bladder paresis have also been reported in a limited number of cases [105, 111-114]. Other frequently reported symptoms among adult LNB patients include meningitis, headache, sleep disturbance, fatigue, malaise, arthralgia, myalgia, paraesthesia, and concentration and/or memory disturbance [76]. Less common peripheral nervous system manifestations involve plexus neuritis and mononeuritis multiplex [115] or brachial plexopathy [116]. In less than 5% of the early LNB cases, central nervous system manifestations occur, such as encephalitis, myelitis and cerebral vasculitis [103].

If symptom duration is longer than 6 months, patients are classified as late LNB [117]. For late LNB cases in Europe, peripheral nervous system involvement include symptoms such as radiculopathy [105], mono- and polyneuropathy [103]. Polyneuropathy is caused by axonal degeneration involving the sensory nerves and has been observed in patients with ACA [103]. Central nervous system involvement among late LNB cases include symptoms such as myelitis, encephalitis [105], encephalomyelitis and cerebral vasculitis [103]. In some cases, a stroke or transient ischaemic attacks was reported [103].

The most common manifestations of LNB among children in Europe are facial nerve palsy and lymphocytic meningitis [118, 119]. Other frequently reported symptoms among European children include fatigue, headache, fever, loss of appetite, neck pain/stiffness, vertigo, radicular pain and EM and/or lymphocytoma (range between 20% to 75%) [108].

In North America, facial palsy is the most common manifestation of LNB (9%), followed by radiculoneuropathy (4%) and meningitis and/or encephalitis (3%) [120].

THE PATHOGENESIS OF LYME BORRELIOSIS

When a blood meal is taken from an infected reservoir host, *B. burgdorferi* s.l. will enter the *Ixodes* tick and migrate to the midgut and remain there until the next tick stage. When an infected tick takes another blood meal, *B. burgdorferi* s.l. multiplies and changes the expression of proteins located on the outer surface. In the midgut, *B. burgdorferi* s.l. expresses OspA, which binds to the tick receptor for OspA that is located on the epithelial cells in the midgut [71, 121]. Through temperature and pH changes during the blood meal, OspA will be downregulated and OspC will be upregulated [71, 122]. Consequently, *B. burgdorferi* s.l. will migrate to the salivary glands [21, 71], where OspC binds to the tick salivary gland protein (Salp)15. With the tick saliva, *B. burgdorferi* s.l. can enter the skin of the mammalian host [71]. Salp15 as well as other substances within the tick saliva have immunomodulatory properties to interfere with the hosts' innate and adaptive immune response and protect *B. burgdorferi* s.l. from complement- and antibody-mediated killing and reduce chemotaxis of immune cells [71, 123-125].

B. burgdorferi s.l. also has various mechanisms to evade the hosts' innate and adaptive immune response [71, 125, 126]. One of the mechanisms to evade the innate immune response is the expression of surface proteins that can interfere with the complement system, such as the fibronectin-binding lipoprotein BBK32 or the CD59-like complement inhibitory molecule [71, 125-130]. *B. burgdorferi* s.l. has also shown to be resistant to antimicrobial peptides (i.e., cathelicidin) [131, 132] and antimicrobial proteins (i.e., lactoferrin, proteinase 3, azurocidin) [133], that are part of the host immune response [125]. In addition, *B. burgdorferi* s.l. elicits the production of anti-inflammatory cytokines by mononuclear cells, such as IL-10, which suppresses phagocytosis and reduces the production of other pro-inflammatory mediators [71, 125, 126, 134]. Furthermore, *B. burgdorferi* s.l. has mechanisms to evade the immune system by hiding in phagocytic cells or in the extracellular matrix [71, 125, 126]. In the extracellular matrix, *B. burgdorferi* s.l. can attach to several proteins such as decorin, which is part of the connective tissue and enables the dissemination of *B. burgdorferi* s.l. and promotes its survival [71]. *B. burgdorferi* s.l. is also suggested by some to be pleomorphic, capable of forming biofilm-like structures [125, 135, 136], or exhibit conformational changes (i.e., round bodies, L-form bacteria, or microcolonies), which may play a role in escaping the immune system [125, 137].

To evade the adaptive immune response, *B. burgdorferi* s.l. can use antigenic variation resulting in the differential expression of surface proteins on the outer membrane [71, 125, 126], such as that

seen in the variable major protein-like sequence, expressed (VlsE) protein [138, 139]. Through recombination events in the Vls locus, the VlsE can change phenotypically. Consequently, this new VlsE protein cannot be recognized by the antibodies already formed. The adaptive immune response is also disabled through invasion of *B. burgdorferi* s.l. into the lymph nodes, as this may disrupt the formation of germinal centers, which are required for the formation of long-lived plasma cells and memory B cells [125, 140]. Furthermore, the strong and sustained IgM response suggests a failure of B cells to undergo a class switch from IgM to IgG [125, 140].

As *B. burgdorferi* s.l. does not have genes encoding toxins [141, 142], most of the clinical symptoms are expected to result from the tissue invasion of *B. burgdorferi* s.l. and the effects of the hosts' innate and adaptive immune response [2].

THE PATHOGENESIS OF LYME NEUROBORRELIOSIS

In 1984, *B. burgdorferi* s.l. was isolated from the CSF of a patient with meningoradiculitis for the first time [143]. Since then, *B. burgdorferi* s.l. has been detected in the CSF of LNB patients by culture and PCR [144]. The presence of *B. burgdorferi* s.l. in the CSF and subsequent activation of local immune cells results in lymphocytic pleocytosis (i.e., an increased number of lymphocytes in the CSF [normal cell count: <5 leucocytes per µl of CSF]), which comprises T cells, B cells, plasma cells and NK cells [145]. The exact location of *B. burgdorferi* s.l. in the central nervous system is unknown; however, using nonhuman primate models, *B. burgdorferi* s.l. has been localized in the leptomeninges, nerve roots, and dorsal root ganglia, but not in the parenchyma [146]. These findings were consistent with the pathological findings among LNB patients with Bannwarth's syndrome [114]. In the peripheral nervous system, *B. burgdorferi* s.l. was found in the endoneurium and in connective tissues of peripheral nerves and muscles using nonhuman primate models [146].

As mentioned previously, *B. burgdorferi* s.l. can enter the CSF via hematogenous dissemination [71], or via dissemination along the peripheral nerves [71, 76], as suggested by several studies which showed that radicular pain is often located in the region of the tick bite and/or EM [76, 147]. Ackermann et al. [148], however, reported no such association. The exact mechanisms that lead to the clinical symptoms found among LNB patients are not entirely clear. In mouse models, *B. burgdorferi* s.l. can bind to glial and neuronal cells, which could be affected by mechanisms such as (in)direct cytotoxicity, or autoimmune reactivity via molecular mimicry causing glial and neuronal cell death [72, 149, 150]. In nonhuman primate models, pro-inflammatory cytokines play a fundamental role in the pathogenesis of LNB [149, 151]. Studies on primary cells of the nervous system and tissue biopsies have shown that the neurotropism of *B. burgdorferi* s.l. and its binding to (but not invasion of) neuronal cells results in the production of inflammatory mediators, which can cause neurological damage [152-155].

THE LABORATORY DIAGNOSIS OF LYME BORRELIOSIS

The diagnosis of LB is mainly based on the presence of clinical findings or symptoms, and, for most Lyme manifestations, laboratory tests are used to support and confirm the clinical diagnosis. For EM, a clinical diagnosis is deemed sufficient to initiate treatment in case of typical lesions, otherwise, laboratory tests must be used to support the diagnosis [2, 156]. Laboratory tests for LB diagnosis can either be based on the direct detection of *B. burgdorferi* s.l., or on the indirect detection of its presence, by demonstrating a host immune response against *B. burgdorferi* s.l..

DIRECT DETECTION METHODS

Direct detection methods include microscopy, culture and PCR. Microscopy in blood or other infected tissues is difficult due to the often low numbers of spirochetes [157]. It is sometimes used for skin, cardiac and synovial tissue examination together with serology; however, for blood and CSF microscopy is not useful [157].

Culture is not routinely used for LB diagnostics due the long growth time, the need of complex media, and the low yield [156, 158, 159]. The yield is best for patients with early, untreated EM and the sensitivity of culture using skin biopsies from EM patients ranges between 40% and 90% [157, 159]. Skin biopsies are mostly taken from the edge of the EM lesion, but can also be taken from the center of the EM lesion as well as from the clinically normal perilesional site of the EM lesion [87, 159, 160]. The sensitivity of skin biopsies of borrelial lymphocytoma amounts to a maximum of 35%, and that of ACA to a maximum of 40% [156, 161, 162]. Blood cultures are mostly positive in the early phase of the infection consistent with the hematogenous spreading of the bacterium at that time, and results are best when large volumes of plasma are used (≥9 ml) [157]. In North America, the sensitivity of plasma culture ranges between 40% and 50%, while in Europe the sensitivity is below 10% [156, 157]. This could be related to the lower rate of multiple simultaneous EM manifestations in Europe [93]. The sensitivity of blood culture of disseminated LB cases and of CSF culture of LNB cases is low in both North America and Europe (<10% and <15%, respectively) [156, 157], and culture of synovial fluid of LA patients has not been successful [157]. This suggests that the bacterium can only be readily detected in early Lyme manifestations, excluding ACA. For all these direct diagnostic approaches, specialized personnel is needed.

PCR can be used to diagnose LB, especially in the early phase of infection, prior to antibody synthesis and antibiotic treatment [159, 163]. Many studies have investigated the use of PCR detection of *B. burgdorferi* s.l. for LB diagnostics, and reported sensitivities varied extensively [159]. This might be explained by the PCR method, the target genes and primers used, and the clinical presentation [159]. Furthermore, much attention should be paid to the sample collection, sample transport and sample processing, as incorrect handling can negatively influence the sensitivity of the PCR [159]. The interpretation of PCR results can be complicated as a negative PCR result does not exclude LB [164], and due to the high sensitivity of PCR, false-positive PCR results may incidentally occur as well [164, 165]. In general, the sensitivity of PCR detection of *B. burgdorferi* s.l. in skin biopsies of EM and ACA patients is high, with a median of 69% (range: 36% to 88%) and 76% (range: 54% to 100%), respectively [159]. An equally high sensitivity was found for PCR detection of *B. burgdorferi* s.l. in synovial fluid of LA patients (median: 78% [range: 42% to 100%]) [159]. The sensitivity of PCR detection of *B. burgdorferi* s.l. in CSF of LNB patients is much lower (median: 38% [range: 12% to 100%]) and the best results are obtained for very early LNB cases [159, 166]. In the presence of lymphocytic pleocytosis, the sensitivity of PCR detection of *B. burgdorferi* s.l. in CSF is usually higher [163, 167]. PCR detection of *B. burgdorferi* s.l. in blood has the lowest sensitivity (median: 14% [range: 0% to 100%]), and is hampered by the low spirochetemia (estimated to approximately 0.1 spirochetes/ml of whole blood) [168] and the dissemination of *B. burgdorferi* s.l. in the tissues of joints, heart and the central nervous system [159, 163]. The specificity of PCR detection of *B. burgdorferi* s.l. was very high (approximately 100%) for all Lyme manifestations [159].

INDIRECT DETECTION METHODS

Indirect detection methods are based on the immune response of the host against *B. burgdorferi* s.l.. The most widely used tests in routine clinical practice to support the diagnosis of LB are based on the detection of *Borrelia*-specific antibodies in blood [169]. Most guidelines recommend the use a two-tier test strategy [86, 156]. This two-tier test strategy aims to improve the diagnostic performance of laboratory tests by combining a highly sensitive first test (i.e., a screening test)

with a highly specific second test (i.e., a confirmation test) to confirm equivocal and positive test results obtained in the first test [169-171]. The screening test often comprises an enzyme-linked immunosorbent assay (ELISA), and the confirmation test is based on either a western blot (North America) or an immunoblot (Europe) [169]. In North America, a modified two-tier test strategy has been approved by the Unites States Food and Drug Administration in which the western blot is substituted for a second ELISA [172, 173]. This modified two-tier test strategy has shown to be at least as sensitive as the conventional two-tier test strategy in diagnosing LB without a loss in specificity [174, 175]. Among patients with EM and early LNB, a single-tier test strategy using an ELISA based on the C6 peptide, which is derived from the immunodominant invariable region 6 of VlsE, has also proven to be effective [176]. The origin, type and number of antigens used in the various antibody assays may differ. These antigens can be based on whole-cell lysates, or on one or several purified native antigen(s), recombinant antigen(s), synthetic peptide(s) or a mixture of these [169]. As different *B. burgdorferi* s.l. genospecies are known to cause human disease in Europe, many European ELISAs use antigens derived from these different pathogenic genospecies [169].

The antibody response against *B. burgdorferi* s.l. is considered to be slow and delayed [177, 178]. The initial response is based on IgM against early antigens, such as p41, OspC, and BBK32 (Table 1), which are expressed by the bacterium in the initial stage to establish an infection [169]. Generally, IgM is detectable within 2 to 4 weeks after the start of infection and peaks after 6 to 8 weeks and then declines [169]. Persistence of IgM, however, also occurs [179-181], and may, at least in part, be explained by cross-reactive auto-antigens or antigens from other microorganisms or environmental factors [182]. The IgG response generally becomes detectable 2 to 6 weeks after the start of infection and peaks after 4 to 6 months and can remain elevated for years [183, 184]. The IgG response often starts with antibodies against VlsE, p41, OspC and BBK32, and is followed by antibodies against p18, p39 and p58 and later by p83/p100 [169].

Table 1. The expression of immunodominant *Borrelia* antigens during various stages of human infection. Table adapted from Talagrand-Reboul et al. [169]. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). Full terms at https://creativecommons.org/licenses/by/4.0/.

Early antigens	Early/late antigens	Late antigens
• OspC (p21-p25, outer surface (lipo)protein C)	• VlsE (p34-35, variable major protein-like sequence, expressed)	• p83/100
• Flagellin (p41)	• DbpA (p17-p18, decorin-binding protein A)	• OspA (p31, outer surface (lipo)protein A)
• BBK32 (fibronectin-binding protein)	• BmpA (p39, *Borrelia* membrane protein A)	• p30
	• OppA-2 (p58, oligopeptide-binding protein)	• p66
	• p14	• p93
	• p28	
	• p43	
	• p45	

Serology is not recommended for EM patients presenting with an expanding, annular, skin rash that is characterized by a bull's-eye rash, as this is pathognomonic. However, if the rash is atypical, the detection of *Borrelia*-specific antibodies can be helpful for diagnosis of LB. In Europe, the sensitivity of the two-tier test strategy among EM patients is moderate (~55%, 95% confidence interval [CI]: 32% to 77%) [169, 185, 186]. The sensitivity of the two-tier test strategy among LNB patients using serum is higher than that seen among EM patients (~87%, 95% CI: 60% to 98%), and among LA and ACA patients, the sensitivity of the two-tier test strategy is highest (~93%, 95% CI: 68% to 100%, and 100%, 95% CI: 77% to 100%, respectively) [169, 185, 186]. In North

America, the sensitivity of the two-tier test strategy is comparable to that in Europe and is ~46% (95% CI: 39% to 54%) for localized LB, ~90% (95% CI: 78% to 95%) for early disseminated LB, and ~99% (95% CI: 96% to 100%) for late disseminated LB [169, 187]. The specificity of the two-tier test strategy is very high (>98%) [169, 185, 187].

THE INTERPRETATION OF SEROLOGY RESULTS

The interpretation of serology results can be complicated. False-negative test results can be obtained in case the antigens present in the assay do not match the antigens expressed by the strain causing disease. This discrepancy can be explained by the intra- and interspecies heterogeneity of *B. burgdorferi* s.l. and/or the antigenic variation used by *B. burgdorferi* s.l. during the course of disease [71, 81, 82, 125, 126, 139, 188-190]. The composition of the expressed antigens in whole-cell lysates of cultured strains also depends on the environment in which these strains were cultured, as some antigens are only expressed in vivo or are lost during multiple culture passages [191, 192]. Consequently, a discrepancy can occur between the antigens applied in the antibody assay and those expressed during an active infection against which the antibodies are formed [159, 193]. As was shown in the previous paragraph, the sensitivity of antibody assays is positively correlated with disease stage. The sensitivity also seems positively correlated with the number of antigens applied in the assay [194, 195], although only to a certain extend [196]. Another complicating factor is the low sensitivity of antibody assays in the very early phase of the infection, which has a biological cause as the antibody response must be build up (Figure 6) [159]. Seroconversion occurs after 2 to 4 weeks and, in case of a negative test result at the initial disease phase, serology is advised to be repeated on a second blood sample taken 2 to 4 weeks later if symptoms persist [159]. As antibiotics can preclude or diminish the activation of the immune response, false-negative test results can be obtained, or a seroconversion from IgM to IgG might not occur if treatment starts before the moment of blood sampling [157, 197-200]. In mice, for instance, an abrogated immune response has been linked to the development of short-lived germinal centers incapable of forming memory B cells and long-lived plasma cells [201]. Absence of *Borrelia*-specific antibodies might also be caused by humoral immunodeficiency as was shown in some case reports [202, 203].

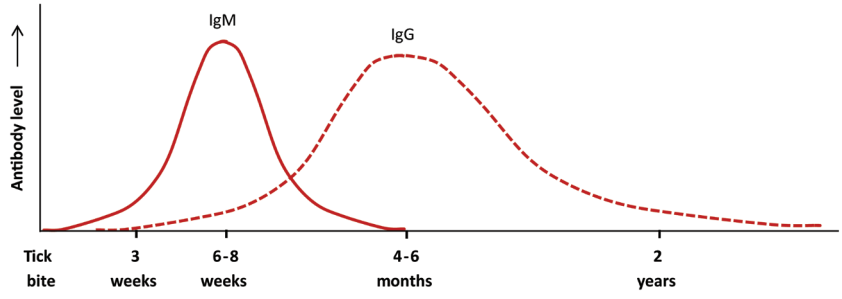

Fig. 6. Antibody response in Lyme borreliosis over time. Figure adapted from Studies in Health Technology and Informatics, Volume 116: Connecting Medical Informatics and Bio-Informatics, Hejlesen OK, Olesen KG, Dessau R, Beltoft I, Trangeled M., Decision Support for Diagnosis of Lyme Disease, 205 – 210, ©2016 [183], with permission from IOS Press. The publication is available at IOS Press through https://ebooks.iospress.nl/volume/connecting-medical-informatics-andbio-informatics.

False-positive test results might be explained by the use of epitopes that are shared by other related spiral micro-organisms, such as *Treponema pallidum*, the causative agent of syphilis [204-206] and various other *Treponema* species [207], or relapsing fever *Borrelia* [208, 209]. False-positive test results have also been reported among patients infected with Epstein-Barr virus [206, 210], cytomegalovirus [206], or *Helicobacter pylori* [206]. Other mechanisms known to cause false-positive (IgM) results are the presence of interfering substances such antinuclear

antibodies or rheumatoid factor [206], cross-reactive auto-antigens or environmental factors [182], or overreading of weak-positive immunoblot bands [211]. Another factor that complicates the interpretation of serology results is the persistence of antibodies after a cleared LB; thus, positive test results do not necessarily indicate active disease [179, 212]. In the literature, reported seroprevalences mostly range between 1% and 20% [213-219], but can be higher in certain regions and among certain risk groups [218, 220-222]. In the Netherlands, an IgG seroprevalence of 9% (regional range: 2% to 17%) was found among blood donors, and an IgG seropositivity of 15% (regional range: 10% to 29%) has been reported among risk groups such as owners of hunting dogs [223] of whom the majority (94%) were asymptomatic. Likely, such seropositivity represents a past infection, and has no (treatment) consequences.

Knowledge regarding the prevalence of LB and the performance characteristics of antibody assays is important in the use of serology for diagnostic purposes (Figure 7) [171]. In general, when serology is performed in a low endemic area, the probability of a positive test result (i.e., the positive predictive value [PPV]) being indicative of active LB is lower than in a high endemic area. In contrast, the probability of a negative test result (i.e., the negative predictive value [NPV]) excluding active LB is lower in a high endemic area than in a low endemic area.

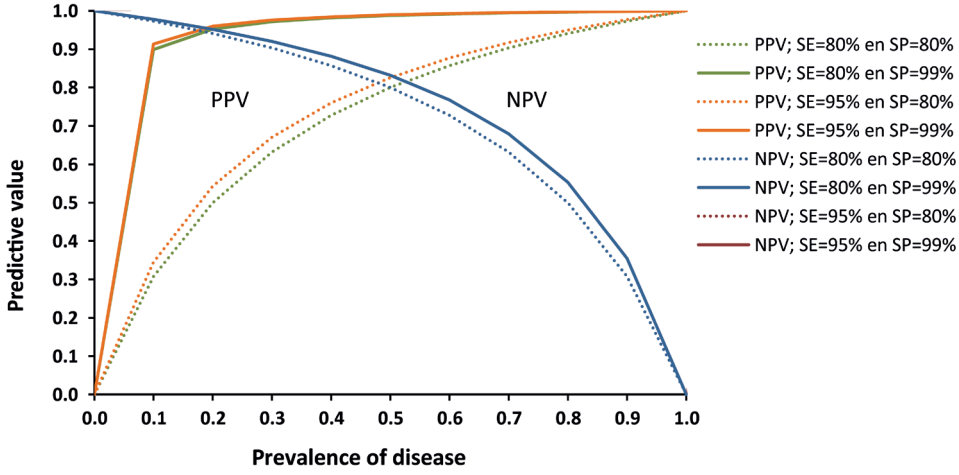

Fig. 7. The relationship between the pre-test probability and the positive- (PPV) and negative predictive value (NPV) of a test used to diagnose a disease [171]. Predictive values are shown using a test with a sensitivity (SE) of 80% (green and blue lines) and 95% (orange and red lines), and a specificity (SP) of 80% (dotted lines) and 95% (solid lines).

THE LABORATORY DIAGNOSIS OF LYME NEUROBORRELIOSIS

To diagnose LNB, clinicians seek to confirm clinical symptoms presumptive for LNB by the findings of (non-) specific immunological changes in the CSF. Non-specific changes in the CSF of LNB cases usually constitute a lymphomonocytic pleocytosis [224], although in a limited number of cases the absence of pleocytosis has been reported [105, 225]. Other non-specific changes in the CSF of LNB patients usually constitute the presence of oligoclonal IgG, the demonstration of intrathecal synthesis of total IgM and/or total IgG, elevated protein levels, and/or a dysfunctional blood-CSF barrier [226, 227]. A dysfunctional blood-CSF barrier is characterized by an increased CSF to serum ratio of albumin compared to the age-related CSF to serum ratio of albumin and reflects a reduced turnover rate of CSF [228]. An increased CSF to serum ratio of albumin can also implicate blood admixture of CSF and can be differentiated from a reduced CSF turnover rate via differential cell counts [228]. A dysfunctional blood-CSF barrier often intensifies during the course of disease

[227], and both the CSF leucocyte count and the blood-CSF barrier functionality will improve when symptoms resolve. Intrathecal synthesis of total IgM and/or total IgG, however, can remain present for a longer period of time [227, 229]. Low CSF glucose levels, which can be indicative for bacterial meningitis, are mostly seen in patients with chronic LNB [230]. Specific changes in the CSF of LNB cases comprise the presence of intrathecally produced *Borrelia*-specific antibodies. Normal CSF findings, however, do not exclude LNB and have been reported in very early cases of LNB and might be linked to an infection with *B. afzelii* [75, 144, 231]. Normal CSF findings are also observed in patients with ACA-associated polyneuropathy in Europe [232] or in patients with chronic neuropathy in North America [233].

The European Federation for Neurological Societies (EFNS) have constructed guidelines for the diagnosis and subsequent classification of LNB patients in definite or possible LNB [115]. A definite LNB patient must fulfill the following three criteria: (i) clinical symptoms suggestive of LNB (such as meningo-radiculitis, unilateral or bilateral facial palsy, other cranial neuropathies, meningitis, encephalitis, myelitis, or vasculitis) in the absence of another explanation for these symptoms, (ii) CSF pleocytosis, and (iii) intrathecal *Borrelia*-specific antibodies synthesis. A possible LNB patient must have clinical symptoms suggestive of LNB and either one of the other two criteria. In the absence of intrathecally produced *Borrelia*-specific antibodies, a possible LNB patient must have *Borrelia*-specific serum antibodies detectable 6 weeks post infection. If symptom duration is less than 6 weeks, then a positive PCR or culture may be supportive. Patients with late polyneuropathy (symptom duration of more than 6 months) can only be classified as a definite LNB patient if they have peripheral neuropathy, ACA and *Borrelia*-specific serum antibodies. In all other cases, patients are classified as non-LNB patient.

In North America, guidelines recommend that the diagnosis of LNB should be supported by the presence of *Borrelia*-specific serum antibodies (in case of peripheral or central nervous system involvement) and/or intrathecal synthesis of *Borrelia*-specific antibodies (in case of central nervous system involvement) [234]. In case the central nervous system is involved, pleocytosis can also support the diagnosis [234].

THE DETECTION OF INTRATHECALLY PRODUCED *BORRELIA*-SPECIFIC ANTIBODIES

In the absence of a gold standard test, the detection of intrathecally produced *Borrelia*-specific antibodies (IgM and IgG) is currently recommended for the diagnosis of LNB [115]. This is based on the measurement of the relative amounts of *Borrelia*-specific antibodies in CSF and serum and the subsequent calculation of a *Borrelia*-specific CSF/serum antibody index (AI) [115, 234]. For LNB diagnostics, one of two methods is often used. The first method is based on the calculation of a *Borrelia*-specific AI by determining the fraction of *Borrelia*-specific antibodies in the CSF and serum as part of the total amount of antibodies in the CSF and serum using the capture ELISA principle as described by Hansen and Lebech [235]. The second method is based on the calculation of a *Borrelia*-specific AI as described by Reiber and Peter [228]. Recently, the detection of *Borrelia*-specific antibodies in CSF only has also been evaluated [236-238].

Ideally, the presence of intrathecally produced *Borrelia*-specific antibodies is determined using methods that consider the functionality of the blood-CSF barrier and discriminate between blood-derived and brain-derived *Borrelia*-specific antibodies in the CSF [227]. Furthermore, methods are preferred that can correct for a poly-specific immune response [227]. Irrespective of the method used (i.e., a capture ELISA [235] or the method described by Reiber and Peter [228]), the calculation of the *Borrelia*-specific AI is complicated, and much attention must be paid to the individual measurements of the *Borrelia*-specific antibodies in the tested CSF/serum pairs, and consequently, to the CSF/serum pair to be used for determining the *Borrelia*-specific AI (Figure 8). When using the method described by Reiber and Peter [228], the relative amounts of total

antibodies in CSF and serum are also determined, and special attention is required with regard to the total antibody CSF/serum quotient to prevent false-negative AI results due to a poly-specific immune response [228].

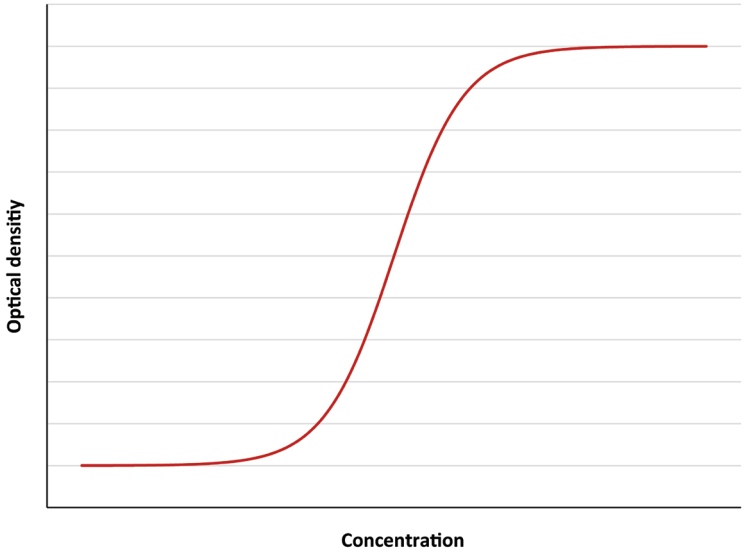

Fig. 8. The relationship between the optical density (OD) values (plotted on the y-axis) and the (log-transformed) concentrations (plotted on the x-axis) of serum and cerebrospinal fluid (CSF) is described by a sigmoid-shaped (S) curve [239]. Ideally, for an antibody index (AI) calculation, the concentrations of both serum and CSF are located in the middle (linear) part of the S-curve. When the OD values of CSF and serum are both located in the lower left corner (or both located in the upper right corner) of the S-curve, small differences between the OD values will result in larger differences between the corresponding concentrations, as is reflected by the almost horizontal lines in the S-curve in both corners [239]. Thus, when the OD values of CSF and serum are both located in the lower left part or upper right part of the curve on the horizontal line, and the serum-OD value is lower than the CSF-OD value, the difference in concentration will be larger, which will result in an increased CSF/serum quotient as well as an increased AI. This can lead to false-positive tests [239]. In contrast, when the serum-OD value is higher than the CSF-OD value, the difference in concentration will be smaller, which will result in a decreased CSF/serum quotient as well as a decreased AI. This can lead to false-negative test results [239]. Therefore, AI test results should be interpreted with care.

Overall, the detection of intrathecally produced *Borrelia*-specific antibodies requires a considerate amount of experience. Therefore, not all diagnostic laboratories are capable to perform such analysis. Furthermore, the interpretation of *Borrelia*-specific AI results is challenging for the same reasons as those encountered with interpreting the serology results performed on blood. Hence, a negative *Borrelia*-specific AI result does not exclude LNB, and a positive *Borrelia*-specific AI result is not an indication of active disease.

THE CELLULAR IMMUNE SYSTEM

Many studies have investigated the usefulness of measuring changes in the cellular immune response for the diagnosis of LB. Some of the diagnostic tools that were studied included the interferon-gamma (IFN-γ) based enzyme-linked immunospot (ELISpot) assay, the lymphocyte proliferation test (LTT), and the B-cell chemokine (C-X-C motif) ligand 13 (CXCL13) ELISA.

Changes in the cellular immune response can be detected by measuring the inflammatory response of the cellular immune system after exposure to pathogen-specific antigens. One of the inflammatory markers is IFN-γ, and for tuberculosis, a number of commercial IFN-γ release assays are available for use in diagnostics [240], among which the IFN-γ ELISpot assay [241]. In this assay,

peripheral blood mononuclear cells from patients are stimulated with antigens derived from *Mycobacterium tuberculosis,* which leads to the production of various inflammatory markers such as IFN-γ by T cells, which can be measured in vitro [242, 243]. For Q-fever, a research group in our hospital has shown the potential of using the cellular immune response in the diagnosis of this disease using an in-house IFN-γ ELISpot assay measuring the antigen-specific T-cell response induced by antigens derived from *Coxiella burnetii* [244], the causative agent of Q-fever. In both tuberculosis and Q-fever, T-cell assays can demonstrate whether a patient is infected; however, differentiation between active disease and a past infection is – as of yet – not possible. Whether this is any different for LB remains to be elucidated.

Before the start of our research into LB diagnostics in 2011, various studies had already shown that the cellular immune response against *B. burgdorferi* s.l. is characterized by a strong T helper (Th)1 response, in which IFN-γ is produced in the blood of patients with LNB and LA [245], in skin lesions of EM patients [246], in synovial fluid of LA patients [247], and in the CSF of LNB patients [248]. Similarly, by using an IFN-γ ELISpot assay, increased numbers of IFN-γ producing *Borrelia*-specific T cells were found in patients with early, late or chronic LB [249-251]. Increased numbers of IFN-γ producing *Borrelia*-specific T cells, however, were also found in seropositive, asymptomatic individuals [252, 253]. Furthermore, it has been put forward that the immune response in LNB patients is mainly localized in the CSF [249, 250, 254, 255], therefore suggesting that the investigation of CSF might be more suitable than that of blood. For LB, however, there are inconsistencies with regard to the results obtained with the IFN-γ ELISpot assay in the studies conducted thus far [256]. Studies often lack cutoff values [256], use different [251, 253, 255, 257], or unknown [258] *Borrelia* antigens and different study populations [256] leading to a large variability in results [253, 256, 257]. Most studies, however, did show that IFN-γ was associated with exposure to *Borrelia* [256].

Other studies reported the use of LTTs for the diagnosis of LB in which the proliferation of T cells is measured upon stimulation with *Borrelia*-specific antigens [259-263]. LTTs for diagnostic purposes are complex in their execution as these often require the use of radioactive substances, prolonged incubation times up to 5 days, and highly specialized personnel. Furthermore, LTTs are less sensitive and less specific than serology [259, 262, 263], although positive LTT results have been found among seronegative patients [259, 260, 263], which might be explained by the (antigen-composition of the) antibody assay used to detect the *Borrelia*-specific antibodies [259, 262]. Overall, studies on LTT showed a large variation in results and lacked clear performance characteristics and LTT is, therefore, not recommended in most LB guidelines [86, 256].

Despite the lack of published studies on clinically validated cellular assays using well-defined study populations, different laboratories offer commercial LTTs or IFN-γ based assays for the routine diagnosis of LB [264-270]. Despite the lack of a robust validation of these tests, patients may be offered prolonged courses of antibiotics assuming that a positive test result for LB is indicative for active disease [270]. This underlines the importance of a thorough clinical validation of these assays using a well-defined study population before implementation in routine diagnostics.

CXCL13 is considered a promising marker for the diagnosis of LNB as elevated levels of this chemokine have been found in the CSF of patients with early LNB, and CSF-CXCL13 levels rapidly decline after antibiotic treatment [271-274]. As illustrated in Figure 9, CXCL13 is produced by mononuclear cells upon entering of *B. burgdorferi* s.l. into the CSF and attracts B cells [71, 275]. Indeed, pleocytosis seen in LNB patients is characterized by a relatively large fraction of B cells [71, 145, 275, 276]. These B cells will mature into plasma cells and subsequently produce *Borrelia*-specific antibodies [71]. This hypothesis is supported by the detection of elevated CSF-CXCL13 levels prior to intrathecal *Borrelia*-specific antibody synthesis [271, 277], thereby showing its

potential for diagnosing early LNB cases. Furthermore, as CSF-CXCL13 levels rapidly decrease after antibiotic therapy, it is a potential marker for measuring disease activity [71, 271-274]. An international reference standard for the use of CXCL13 in CSF is lacking and a broad range of CSF-CXCL13 cutoff levels, ranging from less than 100 to more than 1229 pg/ml, have been published [271, 273, 274, 278, 279]. Thus, appropriate cutoff levels are warranted when CXCL13 is to be implemented for routine LNB diagnostics. However, since elevated CSF-CXCL13 levels are also found in other central nervous system diseases (infectious and non-infectious), results should be interpreted with care [273, 274, 280, 281].

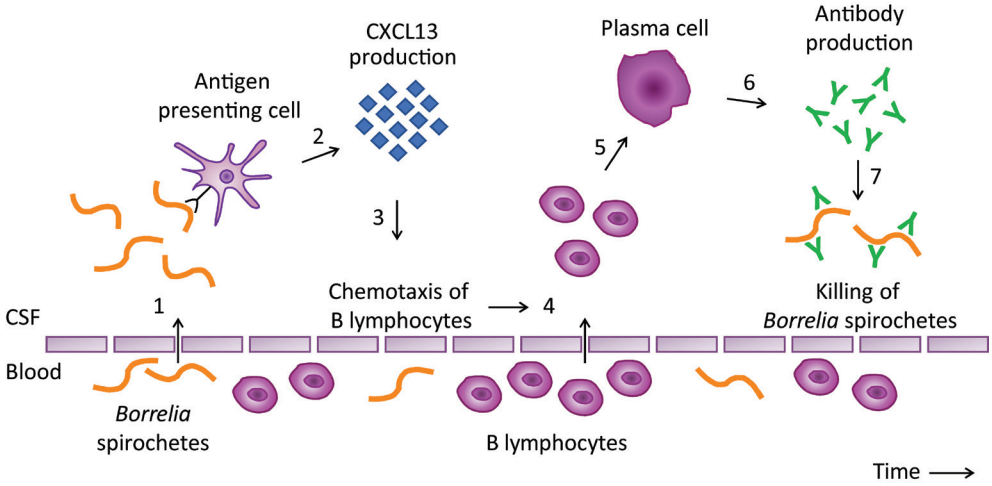

Fig. 9. The antibody response in the CSF in response to a *Borrelia* infection. *B. burgdorferi* s.l. enters the CSF and is recognized by antigen presenting cells (1). These cells subsequently produce CXCL13 (2), which attracts B cells (3). Consequently, B cells migrate into the CSF (4) and mature into plasma cells (5). Plasma cells will, then, produce *Borrelia*-specific antibodies (6) leading to the killing of *B. burgdorferi* s.l. (7). Figure adapted from Rupprecht et al. [71]. This article is published under license to BioMed Central Ltd.. This is an Open Access article distributed under the terms of the Creative Commons Attribution License. Full terms at https://creativecommons.org/licenses/by/2.0.

AIMS OF THIS THESIS

LB is the most commonly reported tick-borne infection and is mainly seen in the temperate regions of the Northern Hemisphere [1]. LB, caused by spirochetes belonging to the *B. burgdorferi* s.l. complex group, is a multisystem disease that can lead to local, early disseminated and late disseminated infection and can involve skin, nervous system, heart, joints and eyes [1]. Except for the typical EM lesions, the diagnosis of LB is based on clinical symptoms and should be confirmed by laboratory tests [86]. The diagnosis of LB, however, can be a challenge due to the large variety of clinical symptoms as these are mostly non-pathognomonic [2]. The variety of clinical symptoms might be caused by the intra- and interspecies heterogeneity of *B. burgdorferi* s.l. and/or host genetic factors [71, 81, 82, 125, 126, 139, 188-190]. The definite diagnosis of LB can also be a challenge due to the difficulty of interpreting laboratory test results, as a gold standard test is lacking. Negative test results do not exclude active disease and positive test results are no proof of active disease.

Due to these challenges for, and uncertainty of LB diagnosis, numerous civilians in North America [282-284] and Europe [285-289], who are affected by LB, have established support groups in a struggle for more recognition for patients with (possible) LB. These patient support groups provide a means to talk to and meet other patients affected by LB and to seek and provide

mutual support, and discuss issues regarding current diagnostics and research. The frustration regarding LB diagnostics among patients has even led to law suits against medical specialists [290]. In 2010, the Dutch Association for Lyme Disease Patients (NVLP) presented a petition to the Dutch Parliament, signed by over 70,000 people, to raise political attention and funding for research to improve diagnostics and treatment of LB, to increase knowledge about LB and its various manifestations, and to establish Lyme treatment centers to better serve patients with LB [291, 292]. Some of the concerns raised by the NVLP involve the use of antibody assays for LB diagnostics, including the lack of standardization of antibody tests, inter-laboratory test variation, and the inability of tests to detect all European *Borrelia* genospecies. Current diagnostic tests lack sensitivity and specificity leading to uncertainty for both medical specialists and patients whether an active infection with *Borrelia* can be excluded. Consequently, Lyme patients may feel unrecognized and seek help elsewhere (outside the main stream clinics) and rely on non-validated tests [264, 265] to 'prove' they have LB and often receive long-term antibiotic treatments [270]. Unfortunately, many of these tests lack proper validation and are not reimbursed by health insurance companies leading to unnecessary patient suffering and high costs [270, 291]. Consequently, the petition of the NVLP involved a number of requests. One of these requests included the development of more sensitive laboratory tests that better reflect the various *B. burgdorferi* s.l. genospecies and take into account the antigenic variation displayed by *B. burgdorferi* s.l.. Two other requests involved (i) the use of multiple tests for the diagnosis of LB, which should include tests based on direct detection methods, and (ii) the acceptance of tests offered abroad as these are currently not reimbursed by insurance companies. As a response on this petition, the Dutch government secured funding for the development of a Lyme expertise center to address these (diagnostic) challenges.

Medical specialists and patients alike are faced with the challenges surrounding LB diagnostics and better diagnostic tools are warranted, especially, since early and correct diagnosis of LB is essential for adequate treatment with antibiotics [21, 76, 293-296]. Therefore, the research in this thesis aims at addressing whether the diagnostic approach for LB (mainly LNB) can be improved and whether active disease can be distinguished from a previous - yet cleared – infection. To answer these questions, current and alternative diagnostic tests and/or algorithms are evaluated that include well-established diagnostic tests based on the humoral immune response (i.e., the detection of *Borrelia*-specific antibodies) as well as alternative diagnostic tests based on the cellular immune response. Consequently, this thesis is divided into two parts; one focusing on the humoral immune response against a *Borrelia* infection, and one focusing on the cellular immune response against a *Borrelia* infection. As clear case-definitions are defined for active LNB, well-defined patients with LNB are used as a proxy for patients with active LB.

OUTLINE OF THIS THESIS

PART I: THE CELLULAR IMMUNE RESPONSE

The research in this thesis, amongst others, involves the evaluation of the IFN-γ ELISpot assay for the routine diagnosis of LNB, as robust validations of such assays using well-defined study populations are lacking [264-270]. As our laboratory has a long-time experience in performing IFN-γ ELISpot assays [244, 297-304], an in-house *Borrelia* ELISpot assay was developed. To evaluate this assay for use in LNB diagnostics, a prospective case-control study (i.e., the T-cell response in Lyme (TRIL)-study) was set-up. The regional Medical Research Ethics Committees United approved the study (Nieuwegein, the Netherlands; MEC-U: NL36407.100.11), and all study participants gave their informed consent. Cases consist of well-defined active LNB patients (i.e., active LNB served as a proxy for active disease), and controls are divided into three groups

and consist of either patients treated for LNB in the past, healthy individuals treated for LB (mainly cutaneous) in the past, or untreated healthy individuals. In **chapter 2**, using the study population of the TRIL-study, the diagnostic potential of the in-house *Borrelia* ELISpot assay is evaluated. Therefore, the number of IFN-γ-producing T cells are measured after stimulating peripheral blood mononuclear cells with *B. burgdorferi* s.s. (strain B31) to investigate whether *Borrelia*-specific IFN-γ-producing T cells can be used as a marker for disease activity. Additional risk factors such as previous tick bites, clinical symptoms, and antibiotic treatment for LB are investigated to assess their contribution to the diagnostic performance of the in-house *Borrelia* ELISpot assay.

In **chapter 3**, the diagnostic performance of a commercial LymeSpot assay, that has not been validated previously, is compared to the diagnostic performance of the in-house *Borrelia* ELISpot assay studied in **chapter 2**. Again, active LNB patients are used as cases and the results are compared with those obtained from controls existing of treated LNB patients, and healthy individuals with or without a history of treated LB (mainly cutaneous).

In **chapter 4**, the diagnostic potential of two commercial CXCL13 assays on CSF is investigated. One of the assays under investigation has been studied extensively for testing CSF, even though the instruction manual for this assay does not mention the use of CSF for the detection of CXCL13. Consequently, this manual lacks a cutoff value for CSF-CXCL13. In the literature, a broad range of cutoff values are mentioned for this assay, which necessitates its validation before use in our hospital. To investigate the diagnostic potential of these two CXCL13 assays on CSF, a retrospective, cross-sectional study design was constructed using a well-characterized study population representative for the clinical setting in which these assays will be used.

PART II: THE HUMORAL IMMUNE SYSTEM

The detection of intrathecally produced *Borrelia*-specific antibodies should be assessed by the detection of these antibodies in CSF and serum within the same run and the subsequent calculation of a *Borrelia*-specific AI. To ensure optimal accuracy, the well-to-well variation (i.e., the intra-assay variation) of the test plate should be minimal. In **chapter 5**, the intra-assay variation of the commercial Enzygnost Lyme link VlsE/IgG ELISA that can be used for the diagnosis of LNB is investigated using a single dilution of the positive kit control (human serum with specific IgG to *B. burgdorferi*). The Enzygnost Lyme link VlsE/IgG ELISA measures IgG to a whole-cell lysate of *B. afzelii* PKo supplemented with recombinant VlsE. The possible impact of the intra-assay variation on LNB diagnostics is investigated through simulation using almost the same study population as the one used in **chapter 4** (i.e., the study population in **chapter 5** comprised 149 of the 156 patients used in **chapter 4**).

In **chapter 6**, the diagnostic performance of seven commercial antibody assays for LNB diagnostics is investigated using the same study population as the one used in **chapter 4**. Five antibody assays were based on the detection of *Borrelia*-specific antibodies (IgM and IgG) in CSF and serum and subsequent calculation of the *Borrelia*-specific (IgM and IgG) AI. Two antibody assays were based on the detection of these antibodies in CSF only as the calculation of a *Borrelia*-specific AI is rather complicated as was mentioned previously. Consequently, not all laboratories have the capacity and expertise to perform these analyses and, thus, a CSF-only assay to prove intrathecal *Borrelia*-specific antibody synthesis is to be preferred. Using multiparameter analysis, various routine CSF parameters (i.e., leucocyte count, total protein, blood-CSF barrier function, and intrathecal total-antibody synthesis) and a number of other parameters (i.e., *Borrelia*-specific serum antibodies, CSF-CXCL13, and a *Borrelia* species PCR on CSF) are investigated to assess their contribution to the diagnostic performance of each antibody assay.

In **chapter 7**, two standard two-tier test strategies for the detection of *Borrelia*-specific antibodies in serum are evaluated. The two strategies are based on an ELISA (either the C6 ELISA or the Serion ELISA), followed by confirmation of equivocal and positive ELISA results using the *recom*Line immunoblot. The C6 ELISA measures total immunoglobulin to the recombinant C6 peptide, and the Serion ELISA measures IgM and IgG to two whole-cell lysates of *B. burgdorferi* s.l. (i.e., *B. afzelii* Pko and *B. garinii)* with the addition of recombinant VlsE for the detection of IgG. A third test strategy is also evaluated and consists of a more unconventional approach based on the combination of both ELISAs as a screening test and immunoblot confirmation of all test results, except concordant negative test results. Again, active LNB patients are used as a proxy for active disease and results are compared to those obtained by testing the serum of treated LNB patients, and healthy individuals with or without a history of treated LB (mainly cutaneous).

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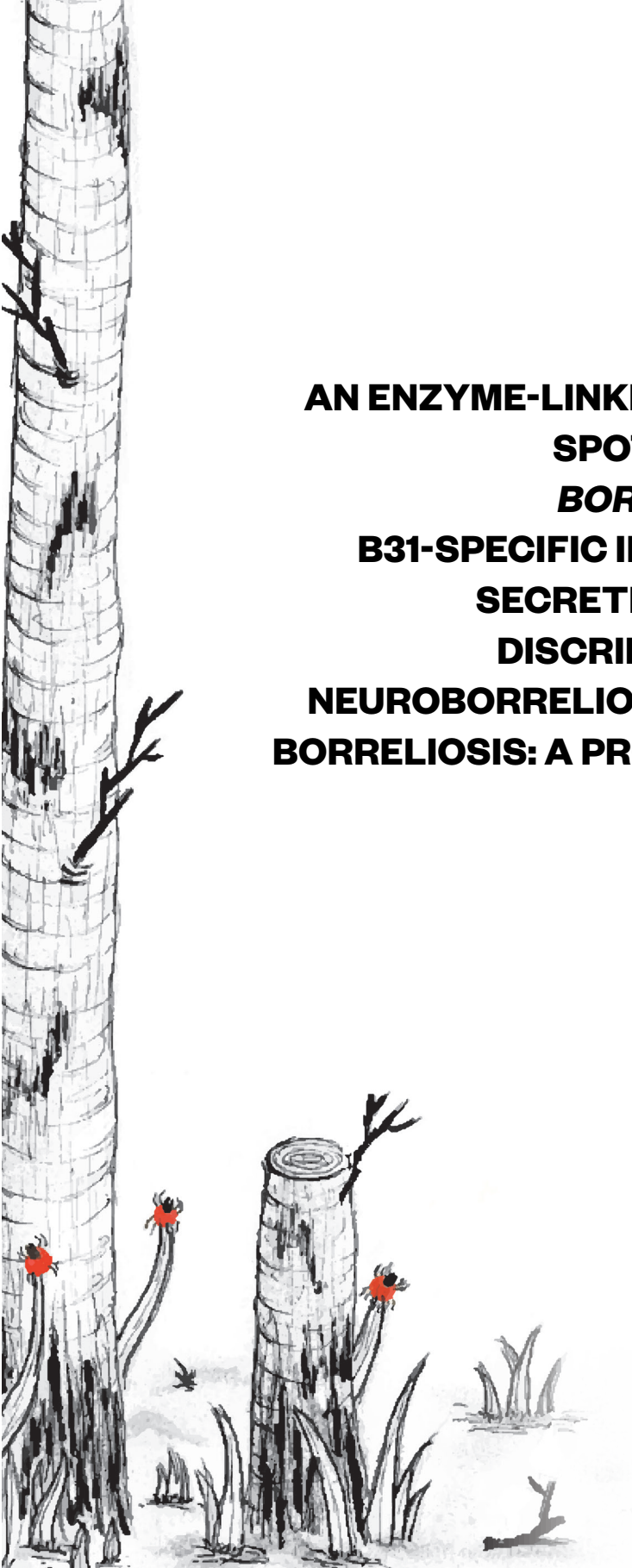

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AN ENZYME-LINKED IMMUNOSORBENT SPOT ASSAY MEASURING *BORRELIA BURGDORFERI* B31-SPECIFIC INTERFERON GAMMA-SECRETING T CELLS CANNOT DISCRIMINATE ACTIVE LYME NEUROBORRELIOSIS FROM PAST LYME BORRELIOSIS: A PROSPECTIVE STUDY IN THE NETHERLANDS

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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 x 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 x 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 x 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/µl) [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 Diakonessenhuis 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 Diakonessenhuis 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 x *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 x *g*; the second wash step was also performed at room temperature for ~7 min at 300 x *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 M NH_4Cl [pH 7.3 ± 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 x 10^6/ml and 100 µl of that concentration was added to a precoated polyvinylidene difluoride (PVDF) ELISpot^{PRO} well (Mabtech, Nacka Strand, Sweden). The negative control consisted of 50 µl of AIM-V medium, and as a positive control, 50 µl (0.1 µg/ml) of anti-human CD3 monoclonal antibody (MAb) CD3-2 (Mabtech) was used. To stimulate the cells, 50 µl of a whole-cell lysate (5 µg/ml), a peptide mix (5 µg/ml), and five recombinant antigens were tested (15 µg/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 *recom*Line 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 µl of 7-B6-1–alkaline phosphatase (ALP) conjugate (Mabtech). The wells were washed again in PBS and incubated with 50 µl 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 (mm^2). 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 ($P \leq 0.041$); 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 ($P \leq 0.015$).
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 intrathecally 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 x 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 x 10^5 PBMCs and the number of *B. burgdorferi* B31-specific IFN-γ-secreting T cells/2.5 x 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 x 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

				Recombinant antigens						*B. burgdorferi* B31 vs recombinant antigen *P* value	
Study groups	**Parameter**	***B. burgdorferi* B31**	**Osp-mix[b]**	***B. burgdorferi* p18 PKa**	***B. afzelii* p18 PKo**	***B. garinii* p18 PBi**	***B. afzelii* p39 PKo**	***B. garinii* p58 PBi**	***B. burgdorferi* B31 vs Osp-mix[b] *P* value**	**Overall**	**2-group**
All study participants	*n*	243	95	119	118	118	118	118			
	%	100	39.1	49.0	48.6	48.6	48.6	48.6			
	Borrelia ELISpot result[a]	3.0	1.0	0.0	0.0	0.0	0.0	1.0	<0.001[e]	<0.001	≤0.047[f]
	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			
Active Lyme NB patients	*n*	33	21	16	16	16	16	16			
	%	100	63.6	48.5	48.5	48.5	48.5	48.5			
	Borrelia ELISpot result[a]	6.0	1.0	0.0	0.0	0.0	0.0	1.5	0.001[e]	<0.001	≤0.020[g]
	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			
Treated Lyme NB patients	*n*	37	13	22	22	22	22	22			
	%	100	35.1	59.5	59.5	59.5	59.5	59.5			
	Borrelia ELISpot result[a]	4.5	0.0	0.0	0.0	0.5	0.0	2.8	0.021[e]	<0.001	<0.001[h]
	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			
Treated healthy individuals	*n*	28	12	14	14	14	14	14			
	%	100	42.9	50.0	50.0	50.0	50.0	50.0			
	Borrelia ELISpot result[a]	7.4	2.0	0.0	1.0	0.0	1.0	1.0	0.028[e]	<0.001	≤0.009[i]
	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			

Table 3. Continued.

Study groups	Parameter	*B. burgdorferi* B31	Osp-mix[b]	Recombinant antigens					*B. burgdorferi* B31 vs Osp-mix[b] *P* value	*B. burgdorferi* B31 vs recombinant antigen *P* value	
				B. burgdorferi p18 PKa	*B. afzelii* p18 PKo	*B. garinii* p18 PBi	*B. afzelii* p39 PKo	*B. garinii* p58 PBi		Overall	2-group
Untreated healthy individuals	*n*	145	49	67	66	66	66	66			
	%	100	33.8	46.2	45.5	45.5	45.5	45.5			
	Borrelia ELISpot result[a]	2	1	0	0	0	0	0	0.017[e]	<0.001	<0.001[j]
	IQR	0.5-3.9	0-1.5	0-1.0	0-1.0	0-1.0	0-1.6	0-1.0			
P value (overall)		<0.001	0.227	0.735	0.311	0.943	0.219	0.002			
P 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 10^5 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 10^5 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 10^5 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 10^5 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 10^5 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 10^5 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 10^5 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 10^5 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 10^5 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 10^5 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 x 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 x 10^5 PBMCs than untreated healthy individuals (median, 2.0; IQR, 0.5 to 3.9) ($P \leq 0.016$) (Table 3).

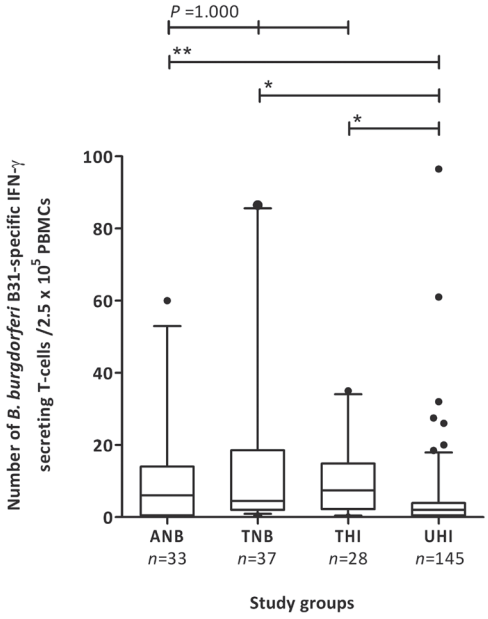

Fig. 1. *B. burgdorferi* B31-specific T-cell activation among active Lyme neuroborreliosis patients, treated Lyme neuroborreliosis patients, treated healthy individuals, and untreated healthy individuals. ANB, active Lyme neuroborreliosis patients; TNB, treated Lyme neuroborreliosis patients; THI, treated 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 x 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).

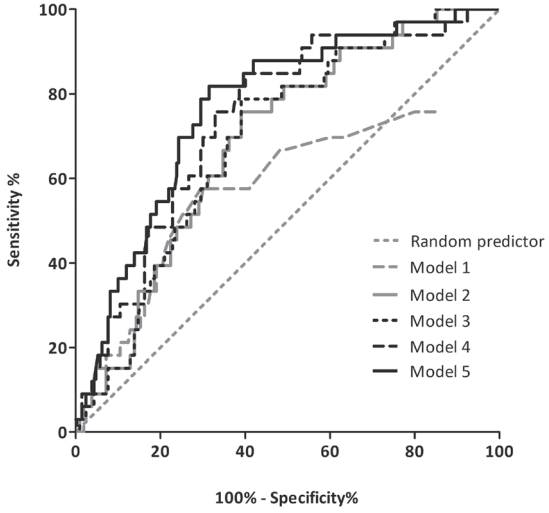

Fig. 2. Receiver operating characteristic (ROC) curve of the *Borrelia* ELISpot assay results and selected logistic regression models that improved the diagnostic performance of the *Borrelia* ELISpot assay used in this study. The ROC curve of model 1 is based on the number of *B. burgdorferi* B31-specific IFN-γ-secreting T cells/2.5 x 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 x 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 x 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 *B. burgdorferi* B31-specific IFN-γ-secreting T cells/2.5x10^5 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 x 10^5 peripheral blood mononuclear cells than seropositive study participants.

BORRELIA ELISPOT ASSAY VERSUS ANTIBODY INDEX FOR ACTIVE LYME NEUROBORRELIOSIS 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 x 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 x 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 x 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 x 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 x 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 x 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 x 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 x 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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SUPPLEMENTAL MATERIAL

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

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

	Logistic regression models				
Covariates logistic regression[a]	**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
Continues variables (*P* values)					
Age		0.012	0.013	0.014	0.001
Borrelia ELISpot result[b]	0.126		0.841	0.872	0.010
Interaction term (*P* values)					
Age by *Borrelia* 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)
Borrelia 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 *Borrelia* 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 x 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).

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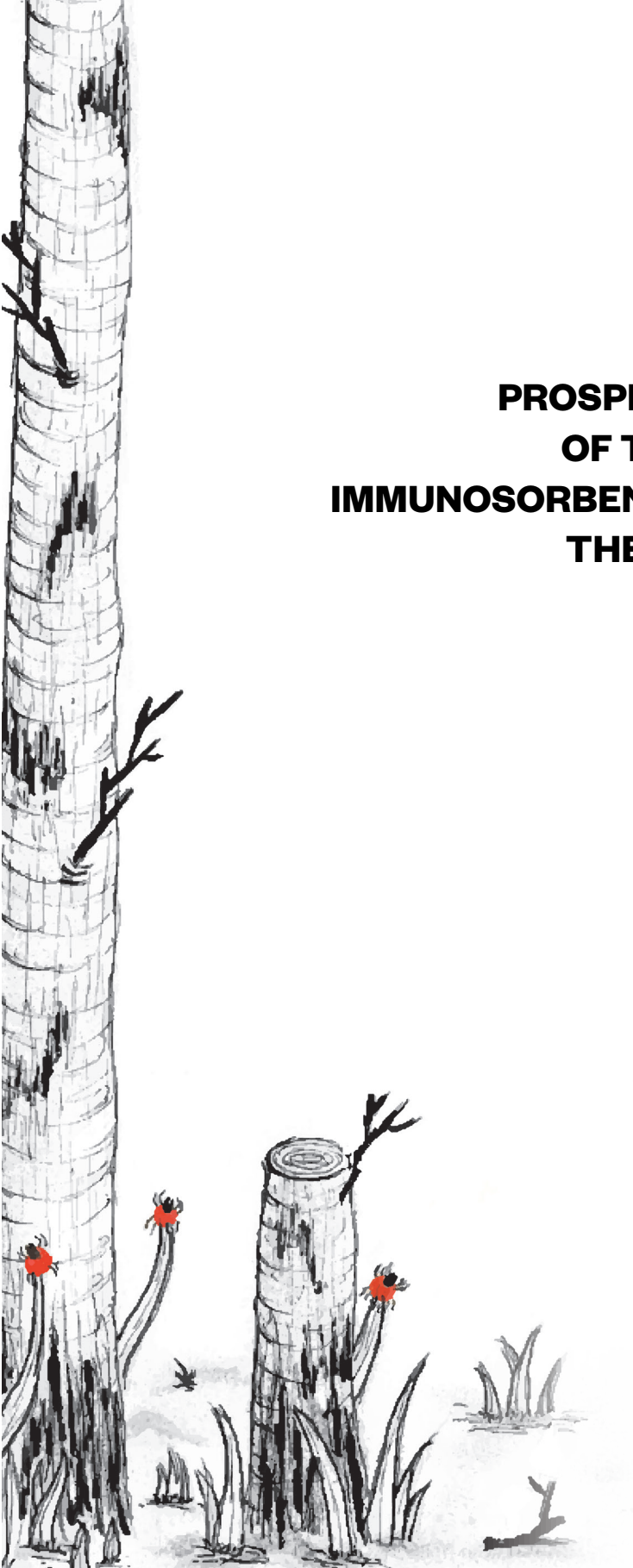

3 PROSPECTIVE COMPARISON OF TWO ENZYME-LINKED IMMUNOSORBENT SPOT ASSAYS FOR THE DIAGNOSIS OF LYME NEUROBORRELIOSIS

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ABSTRACT

Commercial cellular tests are used to diagnose Lyme borreliosis (LB), but studies on their clinical validation are lacking. This study evaluated the utility of an in-house and a commercial enzyme-linked immunosorbent spot (ELISpot) assay for the diagnosis of Lyme neuroborreliosis (LNB). Prospectively, peripheral blood mononuclear cells (PBMCs) were isolated from patients and controls and analysed using an in-house *Borrelia* ELISpot assay and the commercial LymeSpot assay. *B. burgdorferi* B31 whole-cell lysate and a mixture of outer surface proteins were used to stimulate the PBMCs and the numbers of interferon-gamma-secreting T cells were measured. Results were evaluated using receiver operating characteristic (ROC) curve analysis. Eighteen active and 12 treated LNB patients, 10 healthy individuals treated for an early (mostly cutaneous) manifestation of LB in the past and 47 untreated healthy individuals were included. Both assays showed a poor diagnostic performance with sensitivities, specificities, positive and negative predictive values ranging from 44.4-66.7%, 42.0-72.5%, 21.8-33.3% and 80.5-87.0%, respectively. The LymeSpot assay performed equally poorly when the calculation method of the manufacturer was used. Both the in-house and the LymeSpot assay are unable to diagnose active LNB or to monitor antibiotic treatment success.

KEYWORDS
Borrelia, Lyme neuroborreliosis, ELISpot, T cells, interferon-gamma

INTRODUCTION

Lyme borreliosis (LB) is a tick-borne disease caused by bacteria of the *Borrelia burgdorferi* sensu latu group. In Europe, the most prevalent species that cause LB are *B. afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto. The most common manifestation of LB is erythema migrans (EM); other manifestations include Lyme neuroborreliosis (LNB), Lyme arthritis (LA) and acrodermatitis chronica atrophicans (ACA). Most people, however, do not notice any symptoms and clear the infection unknowingly. In the Netherlands, surveys among general practitioners conducted in 1994 and 2017 showed a fourfold increase from an estimated 6,500 to an estimated 25,500 patients with EM [1, 2]. In addition, 1,500 cases of a disseminated manifestation of LB were reported in 2017 [1]. Thus, LB has an increasing impact on public health in the Netherlands [3].

The diagnosis of LB depends on clinical symptoms and can sometimes be difficult due to the lack of a 'gold standard' test, such as culture or polymerase chain reaction (PCR). Culture is only useful for skin manifestations such as EM or ACA, but is not recommended because of the varying sensitivity and long duration, and EM is mainly a clinical diagnosis [4-6]. PCR is particularly useful in skin manifestations and LA [5, 7]. For LNB, both culture and PCR show varying sensitivity and are mostly useful in the early phase of the disease [5, 7-10].

The most frequently used laboratory test for LB is based on the detection of *Borrelia*-specific antibodies. Unfortunately, the interpretation of serological tests can be difficult, as *Borrelia*-specific antibodies can persist lifelong and, hence, do not discriminate between an active LB and a cleared infection. Furthermore, the absence of *Borrelia*-specific antibodies in the early phase of the infection does not exclude LB [11]. Therefore, better diagnostic tools are needed that can establish an active LB, especially because early antibiotic therapy has proved to be effective [12].

In recent years, various cellular assays for the diagnosis of LB have been described. Some of these assays are based on the proliferation of T cells, such as the lymphocyte transformation test (LTT) described by von Baehr et al. [13] or the LTT-memory lymphocyte immunostimulation assay (MELISA) described by Valentine-Thon et al. [14]. Other assays detect cytokines which are secreted by T cells upon stimulation with *Borrelia* antigens, such as the Quantiferon test described by Callister et al. [15] or the enzyme-linked immunosorbent spot (ELISpot) assay (iSpot Lyme) described by Jin et al. [16]. Most studies on cellular assays have used poorly described study populations and lack clinical validations. Despite the lack of such validations, these assays are used in some laboratories for the diagnosis of LB [17-19], and when the test result is positive - thus when *Borrelia*-specific T cells are detected - (long-term) antibiotic treatment regimens are started for treatment of active LB [17], which is of major concern. Therefore, we recently validated an in-house *Borrelia* ELISpot assay for the detection of active LNB on a well-established study population of active LNB patients and various control groups [20]. We concluded that the T-cell activity measured in our in-house *Borrelia* ELISpot assay could not be used as a marker for active LNB. In the current study, we evaluated the diagnostic performance of a commercial LymeSpot assay that has not been validated previously, and compared this to the diagnostic performance of our in-house *Borrelia* ELISpot assay in patients suspected of LNB.

MATERIALS AND METHODS

STUDY POPULATION

Inclusion for this study started in March 2014 and ended in November 2017, and for a large part ran in parallel with two previously published studies [20, 21]. Therefore, most of the study participants in the current study also participated in the previous studies and, hence, the study groups of this study consisted of subgroups of the study groups of these previous studies.

All patients diagnosed with LNB in the Diakonessenhuis Hospital, Utrecht and the St Antonius Hospital, Nieuwegein, the Netherlands, were eligible for inclusion in the study if they fulfilled at least two criteria for LNB as defined by the European Federation of Neurological Societies (EFNS) [10]. These criteria are (i) the presence of neurological symptoms suggestive of LNB without other obvious explanations, (ii) cerebrospinal fluid (CSF) pleocytosis (≥ 5 leukocytes/µl) and (iii) *Borrelia*-specific intrathecal antibody production. If all three criteria were met, then a case was categorized as definite LNB; if two criteria were met, then a case was categorized as possible LNB. Patients were either recently diagnosed with active LNB or had been treated for LNB in the past. Clinical symptoms of LNB patients were classified as cranial or peripheral nerve infections – further divided into radiculopathy, cranial or peripheral neuropathy – or as central nervous system disease (which also included meningoencephalitis). Active LNB patients were recruited from March 2014 to November 2017 and were included if blood was drawn within 2 weeks after the start of antibiotic therapy. Treated LNB patients, who had been diagnosed between September 2006 and September 2014, were enrolled from February 2015 to March 2015 and were included at least 4 months after the completion of antibiotic therapy for LNB. The clinical outcome of both active and treated LNB patients was assessed by a neurologist after antibiotic treatment for active LNB was finished. The clinical outcome was interpreted as either a recovery of clinical symptoms or as no (or incomplete) recovery of clinical symptoms.

Healthy individuals were recruited during the period from March 2014 to December 2015 from personnel of the Diakonessenhuis Hospital, Utrecht, the 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, such as gardens, forests, grasslands and dunes [22]. Thus, the healthy individuals in this study represented a subgroup of healthy individuals, with a high risk of tick exposure. The healthy individuals were further subdivided into two groups. The first group consisted of healthy individuals who had received antibiotic treatment for LB-related symptoms in the past, as they had reported in the Lyme-specific questionnaire, and were referred to as treated healthy individuals. The second group comprised all other healthy individuals and these were referred to as untreated healthy individuals.

All study participants 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 LNB patients was extracted from the hospital information system. Healthy individuals were recruited only if they reported no complaints at the time of inclusion in the study.

ANTIBODY DETECTION IN SERUM AND SERUM–CSF PAIRS

For the detection of *Borrelia*-specific antibodies in serum, the C6 enzyme-linked immunosorbent assay (ELISA) (Immunetics, Boston, MA, USA) was used [23]. Equivocal and positive C6 ELISA results were confirmed using the *recom*Line immunoglobulin (Ig)M and IgG immunoblot test (Mikrogen GmbH, Neuried, Germany) [24]. Detection of intrathecally produced *Borrelia*-specific IgM and IgG antibodies was performed using the second-generation IDEIA LNB test (Oxoid, Hampshire, UK), which was adapted from the original publication by Hansen et al. [25]. Most importantly, the dilution of CSF was adjusted from 1:10 to 1:5, and various incubation times (of patient samples, conjugate and substrate) were shortened. The C6 ELISA and the IDEIA LNB tests were performed using a DS2-automated ELISA instrument (Dynex Technologies, Chantilly, VA, USA) and analyzed with the DS-Matrix™ software (Dynex Technologies). The immunoblot results

were recorded with an automated *recom*Scan system using the *recom*Scan software (Mikrogen GmbH). All assays were performed according to the instructions of the respective manufacturers and were interpreted as described previously [20].

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Isolation of peripheral blood mononuclear cells (PBMCs) was performed from whole blood specimens which were collected in lithium heparin tubes. If isolation of PBMCs started within 8 h after venipuncture, 3 ml of fresh, pre-warmed (37°C) Roswell Park Memorial Institute (RPMI) medium (Life Technologies, Invitrogen, Bleiswijk, the Netherlands) was added to 5 ml of blood and, after gently mixing, transferred into a Leucosep tube (Oxford Immunotec Ltd, Abingdon, UK). PBMCs were separated through density gradient centrifugation (Hettich Rotanta 460 RS; rotor 5624) at room temperature for 15 min at 1000 *g*. If isolation of PBMCs was performed between 8 and 32 h after venipuncture, then a T-Cell Xtend (Oxford Immunotec Ltd) step was performed prior to the addition of 3 ml of RPMI medium and density gradient centrifugation, as previously described [20, 26, 27]. After centrifugation, the PBMC fraction was collected and washed twice in 10 ml RPMI medium. The first wash step was performed at room temperature for 7 min at 600 *g*; the second wash step was also performed at room temperature for 7 min at 300 *g*. If necessary, excess erythrocytes were removed between the first and second wash step using human erythrocyte lysis buffer [0.010 M $KHCO_3$, 0.0001 M ethylenediamine tetraacetic acid (EDTA), 0.150 M NH_4Cl (pH 7.3 ± 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 PBMCs were counted using the AC.T diff 2 analyser (Beckman Coulter, Woerden, the Netherlands), as described previously [20]. After isolation, the PBMCs were adjusted to a concentration of 2.5×10^6/ml using AIM-V medium, 100 µl of which (2.5×10^5 PBMCs) was tested in the in-house *Borrelia* ELISpot assay and the commercial LymeSpot assay [Autoimmun Diagnostika (AID) GmbH, Straßberg, Germany].

THE IN-HOUSE *BORRELIA* ELISPOT ASSAY

The in-house *Borrelia* ELISpot assay was performed as previously described [20]. In brief, a precoated polyvinylidene difluoride (PVDF) ELISpot^{PRO} 96-well plate (Mabtech, Nacka Strand, Sweden) was used, and four wells were tested for each study participant. These wells contained 50 µl of positive control [anti-human CD3 monoclonal antibody (mAb) CD3-2 (0.1 µg/ml); Mabtech], 50 µl of negative control (AIM-V medium), 50 µl of *B. burgdorferi* B31 whole-cell lysate (5 µg/ml; AID), hereafter referred to as *B. burgdorferi* B31, and 50 µl of outer surface protein (Osp)-mix (5 µg/ml; AID), respectively, which were used to stimulate the PBMCs. The Osp-mix consisted of a pool of 9-mer to 11-mer peptides of Osp-A (*B. burgdorferi*, *B. afzelii* and *B. garinii*), native Osp-C (*B. afzelii*) and recombinant p18. For the current study, this protocol was extended by the addition of two wells: the first additional well contained 100 µl of *B. burgdorferi* B31 (5 µg/ml) and the second additional well contained 100 µl of Osp-mix (5 µg/ml) to stimulate the PBMCs (Supporting information, Table S1).

The numbers of *Borrelia*-specific interferon (IFN)-γ-secreting T cells/2.5×10^5 PBMCs (displayed as black spots) were measured with an ELISpot reader (AID) and counted by two different people using the ELISpot software (AID), hereafter referred to as the numbers of spot-forming cells (SFCs). SFCs were counted without prior knowledge of the medical background of the study participants. The SFC size used was based on the expected SFC size of an IFN-γ-producing T cell, as determined by Feske et al. [28], and was set on –2.8 log (mm^2). Samples that had a discrepancy in the numbers of SFCs between the two counting persons were recounted by a third person, whose result was leading. For samples that were stimulated with 50 µl of *Borrelia* antigen, the conditions for recounting have been described previously [20]. For samples which were stimulated with 100 µl

of *B. burgdorferi* B31, a recount was performed for those samples which had a discrepancy in the numbers of SFCs in the critical area (between 0 and 10 SFCs), determined by receiver operating characteristic (ROC) curve analysis. When 100 µl of Osp-mix was used, those samples which had a discrepancy in the numbers of SFCs in the critical area (between 0 and 5 SFCs), determined by ROC curve analysis, were recounted. The results of the in-house *Borrelia* ELISpot assay were only interpreted when the assay was valid; i.e., when the numbers of SFCs upon stimulation in the positive control well were ≥ 20 and in the negative control well were ≤ 6 (the latter representing spontaneous SFCs) (Supporting information, Table S1). If the assay was valid, the final numbers of SFCs in the *Borrelia* antigen-stimulated wells were determined. For the wells containing 50 µl of *Borrelia* antigen, this was performed by subtraction of the numbers of SFCs in the negative control well from the numbers of SFCs in the *Borrelia* antigen-stimulated well. For the wells containing 100 µl of *Borrelia* antigen, the final numbers of SFCs were calculated by first multiplying the numbers of SFCs in the negative control well by 2 before subtracting them from the numbers of SFCs in the *Borrelia* antigen-stimulated well (Supporting information, Table S1). The final numbers of SFCs corresponded with the numbers of SFCs after stimulation with either *B. burgdorferi* B31 or Osp-mix. For some cases, the *Borrelia* antigens were tested several times and, for such cases, the median T-cell count was used to determine the final numbers of SFCs. Using the extended version of our in-house *Borrelia* ELISpot assay, we were able to compare our in-house *Borrelia* ELISpot assay with the LymeSpot assay on the basis of exactly the same (absolute) amount of *Borrelia* antigens (100 µl of a 5 µg/ml concentration per well), as prescribed in the LymeSpot assay protocol. In addition, we could also study the effect of various amounts of *Borrelia* antigen (50 versus 100 µl of a concentration of 5 µg/ml) on the numbers of SFCs for the in-house *Borrelia* ELISpot assay (Supporting information, Table S1).

THE LYMESPOT ASSAY

The LymeSpot assay (AID) was run in parallel with the in-house *Borrelia* ELISpot assay. The LymeSpot assay uses a 96-well PVDF plate coated with anti-human IFN-γ antibodies. The assay was performed according to the manufacturer's protocol (Supporting information, Table S1), except for the isolation of the PBMCs and the amount of PBMCs/well, for which our standard protocol was used as described above and in Supporting information, Table S1. In a pilot study we investigated the influence of this deviation from the LymeSpot protocol, and showed that this had no impact on the diagnostic performance of the LymeSpot assay (see Supporting information, Data S4). Stimulation of the PBMCs in the LymeSpot assay was performed using a negative control (100 µl of AIM-V medium), a positive control (100 µl of Pokeweed; AID), 100 µl of *B. burgdorferi* B31 (5 µg/ml; AID) and 100 µl of Osp-mix (5 µg/ml; AID). Both the *B. burgdorferi* B31 and the Osp-mix antigens were identical to the *Borrelia* antigens used for the in-house *Borrelia* ELISpot assay described above. If the PBMC yield was sufficient, both controls and antigens were tested in duplicate (Supporting information, Table S1).

The final LymeSpot results were only calculated when the assay was valid. Following the manufacturer's instructions, the LymeSpot results were valid when the positive control well had ≥ 50 SFCs and the negative control well had ≤ 10 SFCs. The final LymeSpot results were calculated in two ways. First, the average numbers of SFCs were calculated, similarly as described above for the in-house *Borrelia* ELISpot assay, to allow an objective comparison of the results of the LymeSpot assay with those of the in-house *Borrelia* ELISpot assay (Supporting information, Table S1). Secondly, stimulation indices (SIs) were calculated following the protocol of the manufacturer (Supporting information, Fig. S2). For this, the numbers of SFCs of the negative control needed to be established first. If these numbers were between 3 and 10, SIs were calculated by dividing the numbers of *Borrelia*-specific SFCs by the numbers of SFCs of the negative control. If the numbers of SFCs of the negative control were between 0 and 2, SIs were calculated by dividing the final numbers of *Borrelia*-specific SFCs by 1. The final LymeSpot results were based on the

combination of the results of the SIs of both the *B. burgdorferi* B31 and the Osp-mix antigens, and a case could either be categorized as negative, positive (highly specific), or require diagnostic verification (Supporting information, Fig, S2).

DATA HANDLING AND STATISTICAL ANALYSIS

The results of the in-house *Borrelia* ELISpot assay using 50 µl and using 100 µl of *Borrelia* antigen and the results of the LymeSpot assay were compared with regard to their ability to detect active LNB patients and to distinguish them from the study participants in the other three groups. The 50-µl results were published previously, as part of a larger study population (n = 243) [20]. For both ELISpot assays, a comparison was performed based on the individual, as well as the combined results of the numbers of SFCs after stimulation with either 50 or 100 µl *B. burgdorferi* B31 and 50 or 100 µl Osp-mix. In addition, for the LymeSpot assay, the *B. burgdorferi* B31-specific SI, the Osp-mix-specific SI and the final results based on the combination of both SIs (Supporting information, Fig. S2) were compared between the four study groups. Dichotomous, unrelated data were analyzed using the $\chi 2$ or Fisher's exact test. Quantitative, unrelated data comparing more than two groups were analyzed using the Kruskal–Wallis $\chi 2$ test, and subsequent two-group comparisons were analyzed using the Dunn's test [29]. Quantitative, unrelated data comparing two groups were analyzed using the Wilcoxon rank sum test. Quantitative, related data comparing greater than or equal to two groups were analyzed using the Wilcoxon signed-rank test with continuity correction.

To assess the diagnostic performance of both ELISpot assays, various ROC curves were constructed and used to calculate the area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with 95% confidence intervals (CIs) [30]. The optimal threshold was calculated using the point on each ROC curve for which the distance to the upper left corner (where both sensitivity and specificity are 100%) was shortest, and was determined by the square root of $[(1\text{-sensitivity})^2 + (1\text{-specificity})^2]$. The sensitivity, specificity, PPV and NPV were calculated based on the optimal threshold. For both ELISpot assays, a ROC curve was constructed for each antigen separately by comparing the numbers of *Borrelia*-specific SFCs among active LNB patients with those among the other three groups, as well as for the results of both antigens together. To assess the diagnostic performance of each ELISpot assay using the results of both antigens together, a binomial logistic regression (BLR) model was built before the ROC curves were constructed. The numbers of *B. burgdorferi* B31-specific SFCs and the numbers of Osp-mix-specific SFCs, without and with their interaction term, were included as predictor variables in the model; the outcome variable was binary: sick (all active LNB patients) or not-sick (all other study participants). The performance of the BLR model was assessed by calculating the prediction error using cross-validation. For the LymeSpot assay, ROC curve analysis was also performed based on the SIs after stimulation with *B. burgdorferi* B31 and Osp-mix, as described in the Materials and Methods section covering the LymeSpot assay and in Supporting information, Fig. S2. The final results that needed diagnostic verification were classified as 'positive', and were combined with the positive results. Comparison of the ROC curves was performed using DeLong's test for two correlated ROC curves [30].

Raw P values < 0.05 were interpreted as statistically significant, which were subsequently followed by two-group comparisons where appropriate. To account for the multiple statistical analyses in this study, we applied the Benjamini-Hochberg (BH) procedure and controlled the false discovery rate at the level of 2.5%, i.e., no more than one false positive was allowed to be found in our list of rejections [31]. For all statistical analyses and construction of the figures, Rstudio version 1.1.383, 2009-2017 (Rstudio, Boston, MA, USA) was used.

RESULTS

STUDY POPULATION

Ninety-two study participants were eligible for inclusion in the study; however, five (5.4%) patients were excluded. Four study participants, one active LNB patient, one untreated and two treated healthy individuals, were excluded because of insufficient amounts of PBMCs to perform both ELISpot assays. One untreated healthy individual was excluded because the negative control well in the in-house *Borrelia* ELISpot assay was invalid (> 6 SFCs). Eighty-four (96.6%) of the 87 study participants comprised a subgroup of our recently published study [20]; 77 (88.5%) of the 87 study participants were also part of another published study [21] (Supporting information, Table S3).

CHARACTERISTICS OF THE ACTIVE LNB PATIENTS

Eighteen active LNB patients were included in the study, with a median of 6.0 days after the start of antibiotic treatment for their active disease; their median age was 54.7 years (Table 1). Based on the EFNS criteria [10], 12 of 18 (66.7%) active LNB patients were classified as definite LNB cases and the remaining six (33.3%) were classified as possible LNB cases because of the lack of intrathecally produced *Borrelia*-specific antibodies (Table 1). Clinical symptoms consisted of radiculopathy (n = 2), cranial neuropathy (n = 7) or central nervous system disease (n = 5). Four patients had a combination of different symptoms: one patient had radiculopathy and cranial neuropathy, one patient had radiculopathy, cranial and peripheral neuropathy, one patient had radiculopathy and central nervous system disease and the last patient had cranial neuropathy and central nervous system disease (data not shown). Most active LNB patients had *Borrelia*-specific antibodies in their blood [15 of 18 (83.3%)], which was greater compared to treated LNB patients [one of 12 (8.3%)] and untreated healthy individuals [seven of 47 (14.9%)] (adjusted P value ≤ 0.002) (Table 1). The majority [13 of 18 (72.2%)] of the active LNB patients showed complete recovery after the end of antibiotic therapy for active LNB, which was assessed by the neurologist with a median of 38.0 days after antibiotic treatment ended (Table 1).

CHARACTERISTICS OF THE TREATED LNB PATIENTS

Twelve treated LNB patients were included in the study, who were diagnosed with active LNB on average 5.4 years ago (Table 1). The median age of the treated LNB patients at inclusion was 56.3 years and the majority (91.7%) were classified as definite LNB cases at the time of diagnosis of active LNB in the past. One (8.3%) patient was classified as a possible LNB case because of the absence of pleocytosis (Table 1). Clinical symptoms included radiculopathy (n = 1), cranial neuropathy (n = 4) or central nervous system disease (n = 3). Four treated LNB patients had combined symptomology: one patient had radiculopathy, cranial neuropathy and central nervous system disease, one patient had radiculopathy and cranial neuropathy, one patient had radiculopathy and peripheral neuropathy and one patient had cranial and peripheral neuropathy (data not shown). Ten (83.3%) of the 12 treated LNB patients showed complete recovery after the end of antibiotic therapy for active LNB in the past, which was assessed by the neurologist with a median of 37.0 days after antibiotic treatment ended (Table 1). At inclusion in this study, however, eight (66.7%) of the 12 treated LNB patients reported complaints in the Lyme-specific questionnaire (Table 1). These self-reported symptoms included fatigue, neuropathic complaints, myalgias, arthralgias and cognitive complaints (data not shown).

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

Parameters	Active LNB patients (*n* = 18)	Treated LNB patients (*n* = 12)	Treated healthy individuals (*n* = 10)	Untreated healthy individuals (*n* = 47)	Statistics	
					BH[a] (overall)	BH[a] (2-group)
Gender (n of males; %)	10 (55.6)	7 (58.3)	8 (80.0)	23 (48.9)	0.680	ND[b]
Age (median years; IQR)	54.7 (45.8-63.8)	56.3 (51.2-68.0)	55.2 (41.6-59.5)	35.1 (23.2-44.9)	< 0.001	≤ 0.018[c]
Tick bite (yes; %)	8 (44.4)	8 (66.7)	9 (90.0)	37 (78.7)	0.129	> 0.025[d]
EM (yes; %)	1 (5.6)	3 (25.0)	8 (80.0)[e]	2 (4.3)	0.007	≤ 0.002[f]
Serology (no. of positives; %)	15 (83.3)	1 (8.3)	4 (40.0)	7 (14.9)	0.007	≤ 0.002[g]
Intrathecal *Borrelia*-specific antibody production (no. of positives; %)	12 (66.7)	12 (100)	NA	NA	0.225	NA
Pleocytosis (yes; %)	18 (100)	11 (91.7)	NA	NA	0.687	NA
EFNS criteria						
Definite LNB	12 (66.7)	11 (91.7)	NA	NA	0.462	NA
Possible LNB	6 (33.3)	1 (8.3)				
Time between end of AB and blood sampling (median years; IQR)	NA	5.4 (3.6-6.1)	5.0 (2.0-7.0)	NA	0.888	NA
Time between start of AB and blood sampling (median days; IQR)	6.0 (3.3-7.0)	NA	NA	NA	NA	NA
Recovery[h]						
Time between end of AB and visit at neurologist (median days; IQR)	38.0 (22.5-67.2)	37.0 (15.5-53.0)	NA	NA	0.883	NA
Complete recovery (yes; %)	13 (72.2)	10 (83.3)	NA	NA	0.875	NA
Symptoms at the start of the Study (yes; %)	18 (100)	8 (66.7)	0 (0)	0 (0)	0.007	≤ 0.017[i]

LNB, Lyme neuroborreliosis; *n*, number of study participants; BH, Benjamini–Hochberg; ND, not done; IQR, interquartile range; EM, erythema migrans; AB, antibiotic treatment for Lyme borreliosis (LB); EFNS, European Federation of Neurological Societies [10]; NA, not applicable.

a. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
b. As the initial comparison was not significantly different (raw *P* value > 0.050), two-group comparisons were not performed.
c. Untreated healthy individuals versus treated healthy individuals, treated Lyme neuroborreliosis (LNB) patients and active LNB patients (adjusted *P* values are 0.018, 0.002 and 0.001, respectively).
d. As the initial comparison was significantly different (raw *P* value < 0.050), two-group comparisons were also performed.
e. One treated healthy individual had an atypical skin rash, one had flu-like symptoms after the tick bite.
f. Treated healthy individuals versus untreated healthy individuals and active LNB patients (adjusted *P* values are < 0.001 and 0.002, respectively).
g. Active LNB patients versus treated LNB patients and untreated healthy individuals (adjusted *P* values are 0.002 and < 0.001, respectively).
h. The clinical outcome of both active and treated LNB patients was assessed by the neurologist after antibiotic treatment for active LNB was finished. The clinical outcome was interpreted as either a recovery of clinical symptoms or as no (or incomplete) recovery of clinical symptoms.
i. Treated healthy individuals versus treated and active LNB patients (adjusted *P* values are 0.017 and < 0.001, respectively), and untreated healthy individuals versus treated and active LNB patients (adjusted *P* values are < 0.001 for both).

CHARACTERISTICS OF THE HEALTHY INDIVIDUALS

A total of 57 healthy individuals were included. Ten (17.5%) reported having had antibiotic treatment for an early manifestation of LB in the past, which took place on average 5.0 years ago, and who were therefore classified as treated healthy individuals (Table 1). The median age of the treated healthy individuals was 55.2 years. Nine (90.0%) of the 10 treated healthy individuals reported having had a tick bite, and although this percentage was higher than among the other three groups, it was not statistically significant. Eight (80.0%) of the treated healthy individuals reported an EM, which was higher than among active LNB patients [one of 18 (5.6%)] and untreated healthy individuals [two of 47 (4.3%)] (adjusted *P* value ≤ 0.002) (Table 1). The other two either reported flu-like symptoms or an atypical skin rash after the tick bite.

The remaining 47 (82.5%) healthy individuals all reported never to have had antibiotic treatment for LB, and thus were classified as untreated healthy individuals. Their median age was 35.1 years, which was younger than the other three groups (adjusted *P* value ≤ 0.018) (Table 1).

INFLUENCE OF THE DIFFERENT AMOUNTS OF *BORRELIA* ANTIGEN USED ON THE MEDIAN NUMBERS OF SFCS IN THE IN-HOUSE *BORRELIA* ELISPOT ASSAY

PBMCs of all 87 study participants were stimulated with 50 µl [20] and 100 µl of *B. burgdorferi* B31 and Osp-mix, and subsequently tested in our in-house *Borrelia* ELISpot assay (Table 2). Overall, when 50 µl of *B. burgdorferi* B31 was used to stimulate the PBMCs, a lower median number of SFCs was obtained than when 100 µl of antigen was used (2.0 versus 4.0) (adjusted *P* value < 0.001) (Table 2). When the four study groups were analyzed separately, the association between the use of lower amounts of antigen as stimulant and the lower median number of SFCs remained for untreated healthy individuals (1.5 versus 2.0) (adjusted *P* value = 0.006) (Table 2). Stimulation of PBMCs with either 50 or 100 µl of Osp-mix did not result in a difference between the median numbers of SFCs in the in-house *Borrelia* ELISpot assay (1.0 versus 1.0) (adjusted *P* value = 0.786) (Table 2).

INFLUENCE OF THE DIFFERENT *BORRELIA* ANTIGENS USED FOR PBMC STIMULATION ON THE MEDIAN NUMBERS OF SFCS IN THE TWO ELISPOT ASSAYS

Analysis of the results of all 87 study participants showed that PBMC stimulation with 50 µl of *B. burgdorferi* B31 resulted in a higher median number of SFCs than stimulation with 50 µl of Osp-mix in the in-house *Borrelia* ELISpot assay (2.0 versus 1.0) (adjusted *P* value < 0.001) (Table 2). These results are similar to the results we have published previously using a study population of 243 study participants [20]. When the four study groups were analyzed separately, the median numbers of *B. burgdorferi* B31-specific SFC counts were higher compared to the median numbers of Osp-mix-specific SFC counts, although not significant (adjusted *P* values > 0.025) (Table 2). A higher median number of SFCs after PBMC stimulation with *B. burgdorferi* B31 compared to PBMC stimulation with Osp-mix was also seen when a volume of 100 µl of *Borrelia* antigen was used in the in-house *Borrelia* ELISpot assay (4.0 versus 1.0) (adjusted *P* value < 0.001) (Table 2). Comparisons within each of the four groups showed that this difference remained significant for active LNB patients (adjusted *P* value = 0.017) (Table 2).

In the LymeSpot assay, the higher yield of the *B. burgdorferi* B31 over the Osp-mix remained when the median numbers of SFCs were compared (5.0 versus 1.5) (adjusted *P* value < 0.001) (Table 2). When the four study groups were analysed separately, *B. burgdorferi* B31 remained superior in the LymeSpot assay for untreated healthy individuals and active LNB patients (adjusted *P* values ≤ 0.005) (Table 2).

COMPARISON OF THE MEDIAN NUMBERS OF SFCS IN THE TWO ELISPOT ASSAYS BETWEEN THE FOUR STUDY GROUPS AFTER STIMULATION OF THE PBMCS WITH *B. BURGDORFERI* B31

The PBMCs of treated healthy individuals were stimulated the most when either 50 µl of *B. burgdorferi* B31 was used in the in-house *Borrelia* ELISpot assay or 100 µl of *B. burgdorferi* B31 was used in the LymeSpot assay. The PBMCs of treated LNB patients were stimulated the most when 100 µl of *B. burgdorferi* B31 was used in the in-house *Borrelia* ELISpot assay (Table 2; Fig. 1a,c,e). In contrast, the PBMCs of untreated healthy individuals were stimulated the least, irrespective of the volume and the ELISpot assay used. An increased T-cell activation for patients and treated healthy individuals after PBMC stimulation with *B. burgdorferi* B31 was also seen in our previous study, which included more study participants (n = 243), and suggests that the ELISpot activity is related to exposure to the *Borrelia* bacterium [20].

Analysis of the different amounts of *B. burgdorferi* B31 showed that when 50 µl was used to stimulate the PBMCs in the in-house *Borrelia* ELISpot assay, the median number of SFCs of 1.5 for untreated healthy individuals was lower compared to the median number of SFCs of 9.3 for treated healthy individuals (adjusted P value = 0.015) (Table 2, Fig. 1a). When 100 µl of *B. burgdorferi* B31 was used to stimulate the PBMCs, no differences were found between the four study groups for the in-house *Borrelia* ELISpot assay (adjusted P values > 0.025) (Table 2, Fig. 1c). For the LymeSpot assay, the results were only significantly different between untreated (less ELISpot activity) and treated healthy individuals (more ELISpot activity) (adjusted P value = 0.014) (Table 2, Fig. 1e).

Overall, no difference was found between the median numbers of SFCs between both ELISpot assays when 100 µl of *B. burgdorferi* B31 was used to stimulate the PBMCs (adjusted P value = 0.360). Similarly, no differences were found when the four study groups were analyzed separately (adjusted P values > 0.025) (Table 2).

Table 2. Comparison of the ELISpot results expressed in the numbers of spot-forming cells for the in-house *Borrelia* ELISpot assay and the LymeSpot assay among the four study groups

			SFC count (median; IQR)			Statistics			
			In-house *Borrelia* ELISpot assay		LymeSpot assay	50 µl in-house *Borrelia* ELISpot *versus* 100 µl in-house *Borrelia* ELISpot		100 µl in-house *Borrelia* ELISpot *versus* 100 µl LymeSpot assay	
Study participants	***Borrelia* antigen**		**50 µl**	**100 µl**	**100 µl**	**BH[a] (overall)**	**BH[a] (within each group)**	**BH[a] (overall)**	**BH[a] (within each group)**
All study participants (*n* = 87)	*Bb* B31		2.0 (0.8-6.0)	4.0 (2.0-9.5)	5.0 (2.0-10.3)	<0.001	0.006[b]	0.360	>0.025
	Osp-mix		1.0 (0.0-2.0)	1.0 (0.0-3.0)	1.5 (0.5-3.3)	0.786	>0.025	0.685	>0.025
Active LNB patients (*n* = 18)	*Bb* B31		5.5 (1.3-7.8)	6.5 (1.5-11.5)	6.1 (2.6-10.2)				
	Osp-mix		0.0 (0.0-4.8)	1.0 (0.0-3.0)	1.3 (0.1-2.5)				
Treated LNB patients (*n* = 12)	*Bb* B31		5.5 (2.0-9.9)	9.5 (5.0-17.5)	8.4 (2.4-12.8)				
	Osp-mix		0.5 (0.0-4.5)	0.5 (0.0-2.5)	1.5 (0.9-3.3)				
Treated healthy individuals (*n* = 10)	*Bb* B31		9.3 (3.5-27.0)	6.5 (3.3-22.2)	26.1 (7.5-73.7)				
	Osp-mix		2.0 (1.1-6.0)	2.5 (0.5-7.8)	5.6 (3.3-12.0)				
Untreated healthy individuals (*n* = 47)	*Bb* B31		1.5 (0.0-3.0)	2.0 (1.0-5.5)	3.0 (1.4-6.3)				
	Osp-mix		1.0 (0.0-1.5)	1.0 (0.0-2.5)	1.0 (0.0-2.5)				
Statistics	Bb B31 *versus* Osp-mix	BH[a] (overall)	< 0.001	< 0.001	< 0.001				
		BH[a] (within each group)	> 0.025	0.017[c]	≤ 0.005[d]				
	Bb B31	BH[a] (overall)	0.018	0.132	0.075				
		BH[a] (two-group)	0.015[e]	> 0.025[f]	0.014[e,f]				
	Osp-mix	BH[a] (overall)	0.489	0.766	0.058				
		BH[a] (two-group)	ND[g]	ND[g]	0.005[e,f]				

SFC, spot-forming cell; IQR, interquartile range; ELISpot, enzyme-linked immunosorbent spot; BH, Benjamini–Hochberg; *n*, number of study participants; *Bb* B31, *Borrelia burgdorferi* B31; Osp, outer surface protein; ND, not done; LNB, Lyme neuroborreliosis.

a. To correct for multiple comparisons, the Benjamini-Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
b. The numbers of SFCs among untreated healthy individuals were significantly higher after stimulation with 100 µl of *B. burgdorferi* B31 compared to 50 µl of *B. burgdorferi* B31.
c. The numbers of SFCs after stimulation with 100 µl of *B. burgdorferi* B31 among active Lyme neuroborreliosis (LNB) patients were significantly higher compared to stimulation with 100 µl of Osp-mix.
d. The numbers of SFCs after stimulation with 100 µl of *B. burgdorferi* B31 among untreated healthy individuals and active LNB patients were significantly higher compared to stimulation with 100 µl of Osp-mix (adjusted *P* values are <0.001 and 0.005, respectively).
e. Untreated versus treated healthy individuals.
f. As the initial comparison was significantly different (raw *P* value <0.050), two-group comparisons were also performed.
g. As the initial comparison was not significantly different (raw *P* value >0.050), two-group comparisons were not performed.

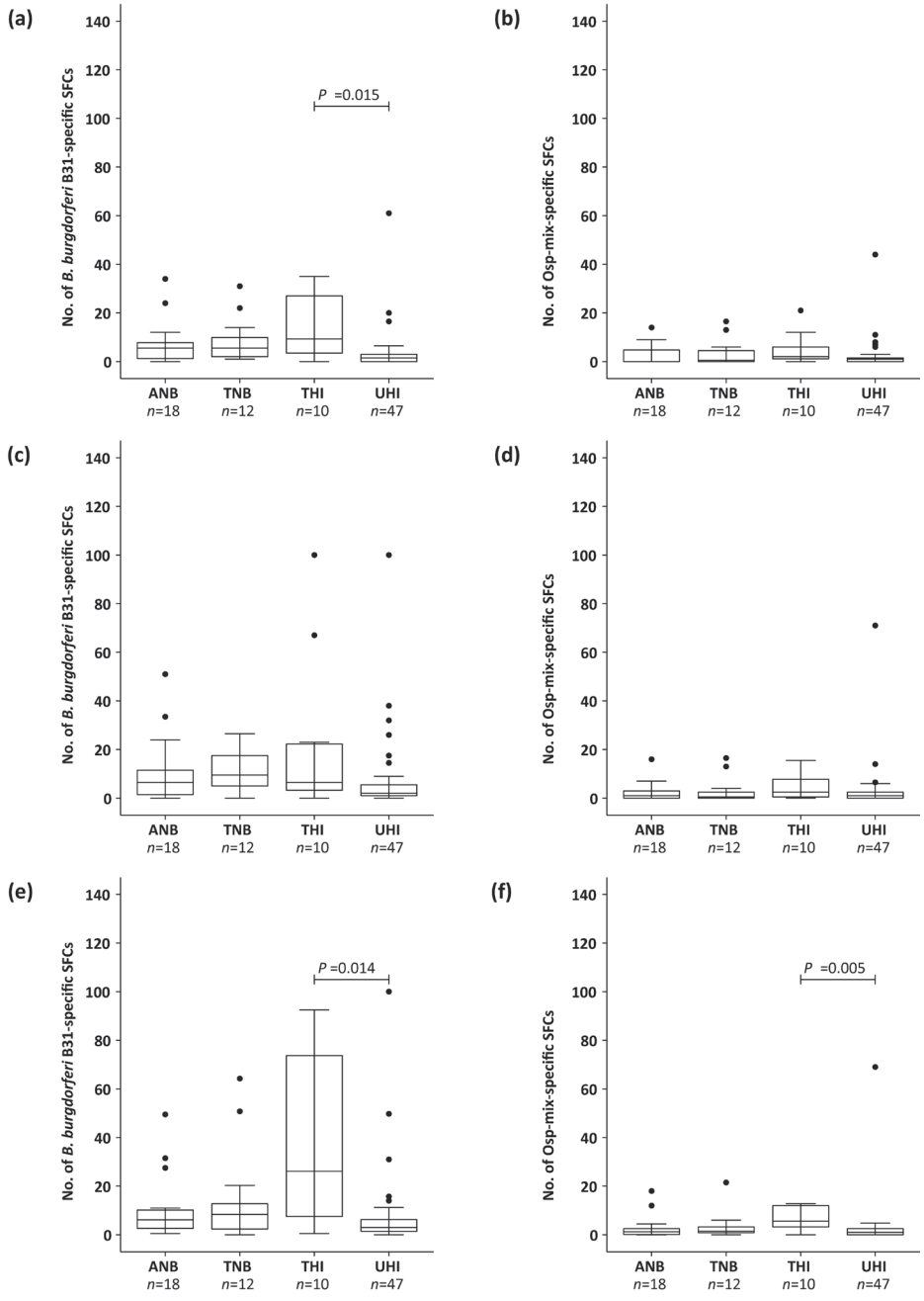

Fig. 1. Results of the in-house *Borrelia* enzym-linked immunosorbent spot (ELISpot) assay (a–d) and the LymeSpot assay (e–f) expressed in the numbers of spot-forming cells (SFCs). (a) (50 µl), (c) and (e) (both 100 µl) are the results after peripheral blood mononuclear cell (PBMC) stimulation with *Borrelia burgdorferi* B31, and (b) (50 µl), (d) and (f) (both 100 µl) are the results after PBMC stimulation with outer surface protein (Osp)-mix among active Lyme neuroborreliosis patients (ANB), treated Lyme neuroborreliosis patients (TNB), treated healthy individuals (THI) and untreated healthy individuals (UHI). The displayed *P* values are corrected and interpreted using the Benjamini–Hochberg procedure with a false discovery rate of 2.5% for multiple comparisons (only false discovery rates < 0.025 are displayed).

COMPARISON OF THE MEDIAN NUMBERS OF SFCS IN THE TWO ELISPOT ASSAYS BETWEEN THE FOUR STUDY GROUPS AFTER STIMULATION OF THE PBMCS WITH OSP-MIX

Similar to stimulation with *B. burgdorferi* B31, the PBMCs of treated healthy individuals were activated most upon stimulation with Osp-mix (Table 2, Fig. 1b,d,f). No significant differences between the four study groups were found for the in-house *Borrelia* ELISpot assay using 50 or 100 µl of Osp-mix (adjusted *P* values 0.489 and 0.766, respectively) (Table 2, Fig. 1b,d). For the LymeSpot assay, however, stimulation of the PBMCs with 100 µl of Osp-mix resulted in a significantly higher median number of SFCs of 5.6 for treated healthy individuals compared to the median number of SFCs of 1.0 for untreated healthy individuals (adjusted *P* value = 0.005) (Table 2, Fig. 1f).

Similar to the use of 100 µl of *B. burgdorferi* B31, no difference was seen between the median numbers of SFCs between both ELISpot assays upon stimulation of the PBMCs with 100 µl of Osp-mix (adjusted *P* value = 0.685). Subsequent comparisons within each group also did not show a difference (adjusted *P* values > 0.025) (Table 2).

THE DIAGNOSTIC PERFORMANCE OF THE TWO ELISPOT ASSAYS BASED ON THE NUMBERS OF SFCS

The diagnostic performance of the in-house *Borrelia* ELISpot assay and the LymeSpot assay were evaluated using ROC curve analysis, for which the numbers of SFCs were used. In order to enable a fair comparison between the two assays, the results obtained with PBMCs that were stimulated with 100 µl of *Borrelia* antigen were used for the in-house *Borrelia* ELISpot assay and compared with the results of the LymeSpot assay. The results obtained with 100 µl of *Borrelia* antigen were used, as this is the standard in the LymeSpot assay (Supporting information, Table S1). ROC curves were constructed based on the results obtained after PBMC stimulation with the *B. burgdorferi* B31 and the Osp-mix separately, as well as on the combined results of both *Borrelia* antigens. The calculated AUCs based on the individual *Borrelia* antigens were comparable to a random predictor, and ranged from 0.459 to 0.570 (Table 3, Fig. 2a,b). No difference was found between the AUC of the in-house *Borrelia* ELISpot assay and the AUC of the LymeSpot assay based on the numbers of *B. burgdorferi* B31-specific SFCs (AUC = 0.553 and 0.570, respectively) (adjusted *P* value = 0.974) (Table 3, Fig. 2a). Similarly, comparison of the AUCs from the two ELISpot assays based on the numbers of Osp-mix-specific SFCs also showed no difference (AUC = 0.479 for the in-house *Borrelia* ELISpot assay and AUC = 0.459 for the LymeSpot assay, respectively) (adjusted *P* value = 0.930) (Table 3, Fig. 2b).

Calculation of the optimal thresholds for the two assays using a single *Borrelia* antigen showed that the sensitivity and NPV was highest for the LymeSpot assay when *B. burgdorferi* B31 was used to stimulate the PBMCs (sensitivity = 66.7%, NPV = 87.0%) (Table 3). The specificity was highest for the in-house *Borrelia* ELISpot assay irrespective of whether *B. burgdorferi* B31 or Osp-mix was used to stimulate the PBMCs (66.7% each) (Table 3). The PPV was highest for the in-house *Borrelia* ELISpot assay when *B. burgdorferi* B31 was used to stimulate the PBMCs (30.6%) (Table 3).

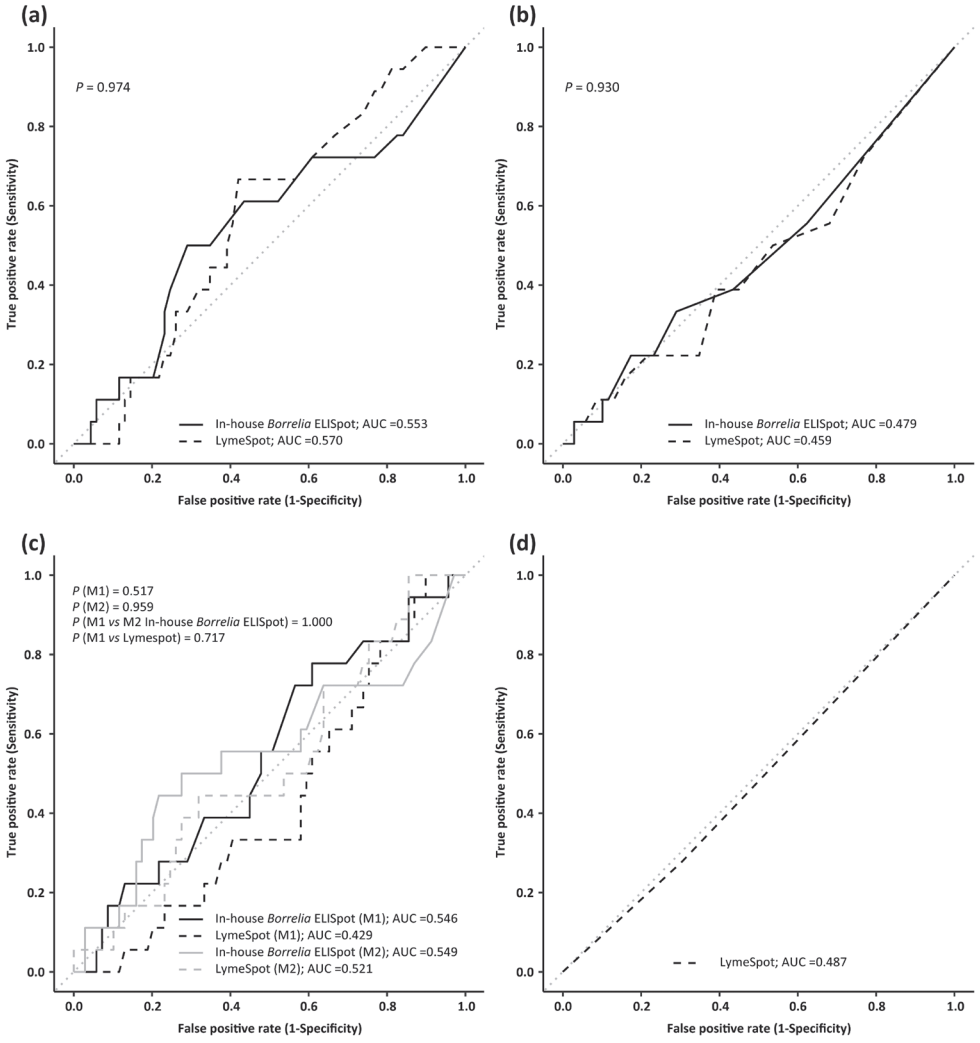

Fig. 2. The receiver operating characteristic (ROC) curves for both the in-house *Borrelia* enzyme-linked immunosorbent spot (ELISpot) assay (solid lines) and the LymeSpot assay (dashed lines) to discriminate active Lyme neuroborreliosis (LNB) patients from the other three groups. The dotted grey line represents the random predictor. (a) ROC curves based on the numbers of spot-forming cells after stimulation with 100 µl of *B. burgdorferi* B31. (b) ROC curves based on the numbers of spot-forming cells after stimulation with 100 µl of Osp-mix. (c) ROC curves based on the outcomes of the two binary logistic regression models (M) for which the combined results of both *Borrelia* antigens, which were based on the numbers of spot-forming cells, without (M1) and with (M2) their interaction term, were included as risk factors. *P* (M1) represents the adjusted *P* value for the comparison of both assays using the outcomes of model 1, *P* (M2) represents the adjusted *P*-value for the comparison of both assays using the outcomes of model 2, *P* (M1 versus M2 in-house *Borrelia* ELISpot) represents the adjusted *P* value for the comparison of the outcomes of models 1 and 2 for the in-house *Borrelia* ELISpot assay, *P* (M1 versus M2 LymeSpot) represents the adjusted *P* value for the comparison of the outcomes of models 1 and 2 for the LymeSpot assay. (d) ROC curve of the LymeSpot assay based on the final LymeSpot result (a combination of the stimulation indices of both antigens following the protocol of the manufacturer (Supporting information, Fig. S2)).

Table 3. Diagnostic performance of the in-house *Borrelia* ELISpot assay and the LymeSpot assay based on the numbers of spot-forming cells obtained after peripheral blood mononuclear cell stimulation with *Borrelia burgdorferi* B31 and Osp-mix separately, and based on the combined numbers of spot-forming cells of both *Borrelia* antigens by using a binomial logistic regression model

Interpretation	ELISpot assay	Threshold[a] (95% CI)	AUC (95% CI)	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)	PPV (%; 95% CI)	NPV (%; 95% CI)	BH[b]	Prediction error BLR model (%)
SFC count after stimulation with *Bb* B31 (100 µl)	In-house *Borrelia* ELISpot assay	7.0 (2.5-8.5)	0.553 (0.382-0.714)	61.1 (38.9-77.8)	66.7 (42.0-81.2)	30.6 (20.5-44.8)	85.7 (79.1-92.2)	0.974	
	LymeSpot assay	5.3 (5.0-8.4)	0.570 (0.433-0.706)	66.7 (44.4-88.9)	59.4 (44.9-72.5)	29.7 (21.1-39.5)	87.0 (79.6-94.7)		
SFC count after stimulation with Osp-mix (100 µl)	In-house *Borrelia* ELISpot assay	2.5 (0.5-5.5)	0.479 (0.326-0.631)	44.4 (16.7-72.2)	66.7 (33.3-87.0)	23.3 (13.8-40.0)	80.9 (72.2-87.1)	0.930	
	LymeSpot assay	2.3 (0.3-4.3)	0.459 (0.305-0.614)	50.0 (22.2-77.8)	56.5 (24.6-84.1)	22.2 (14.6-36.4)	80.5 (72.3-87.5)		
SFC count of both antigens without interaction term in a BLR model[c,d]	In-house *Borrelia* ELISpot assay	0.21 (0.20-0.22)	0.546 (0.398-0.694)	66.7 (38.9-88.9)	49.3 (33.3-81.2)	26.5 (20.0-40.0)	85.7 (78.4-94.3)	0.517	21.8
	LymeSpot assay	0.21 (0.21-0.22)	0.429 (0.292-0.566)	61.1 (27.8-88.9)	42.0 (20.3-73.9)	21.8 (16.1-29.8)	80.8 (72.7-90.6)		21.8
SFC count of both antigens with interaction term in a BLR model[c,d]	In-house *Borrelia* ELISpot assay	0.21 (0.19-0.22)	0.549 (0.380-0.719)	55.6 (33.3-77.8)	72.5 (42.0-85.5)	33.3 (21.1-50.0)	85.7 (79.6-91.8)	0.959	20.7
	LymeSpot assay	0.21 (0.19-0.22)	0.521 (0.372-0.670)	50.0 (33.3-83.3)	68.1 (30.4-81.2)	27.6 (18.9-41.7)	83.9 (77.4-91.3)		20.7
Final result based on the stimulation indices of both antigens	LymeSpot assay	NA[e]	0.487 (0.367-0.606)	27.8 (11.1-50.0)	69.6 (58.0-79.7)	19.1 (7.7-32.0)	78.6 (73.7-84.0)	NA	

ELISpot, enzyme-linked immunosorbent spot; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; BH, Benjamini-Hochberg; BLR, binomial logistic regression; SFC, spot-forming cell; *Bb* B31, *B. burgdorferi* B31; Osp, outer surface protein; NA, not applicable.

a. The threshold is based on the numbers of spot-forming cells when the result of a single *Borrelia* antigen was analyzed, and on the linear predictors of the binary logistic regression model when the combined results of both *Borrelia* antigens were analyzed.
b. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
c. No significant difference (adjusted *P* value = 1.000) between the outcome of the BLR model without interaction term and the outcome of the BLR model with interaction term for the in-house *Borrelia* ELISpot assay.
d. No significant difference (adjusted *P* value = 0.717) between the outcome of the BLR model without interaction term and the outcome of the BLR model with interaction term for the LymeSpot assay.
e. See Supporting information, Fig. S2.

Combining the results of the two *Borrelia* antigens without or with their interaction term as risk factors in a BLR model also resulted in AUCs that were comparable to a random predictor (range = 0.429-0.549) (Table 3). Comparison of the AUCs for the in-house *Borrelia* ELISpot assay and the LymeSpot assay without their interaction term did not show a difference (AUC = 0.546 and 0.429, respectively) (adjusted *P* value = 0.517) (Table 3, Fig. 2c). Similarly, the AUCs for the in-house *Borrelia* ELISpot assay and the LymeSpot assay with their interaction term were also comparable (AUC = 0.549 and 0.521, respectively) (adjusted *P* value = 0.959) (Table 3, Fig. 2c). For both ELISpot assays, the prediction errors of the BLR models with the interaction term were only slightly better than the prediction errors of the BLR models without the interaction term (20.7% for both versus 21.8% for both) (Table 3). Thus, approximately one in five patients were wrongly diagnosed by using the BLR models.

Furthermore, no differences were found between the AUCs of both BLR models for the in-house *Borrelia* ELISpot assay (adjusted *P* value = 1.000) as well as for the LymeSpot assay (adjusted *P* value = 0.717) (Table 3, Fig. 2c). The highest sensitivity was found for the in-house *Borrelia* ELISpot assay when both antigens without their interaction term were included in the BLR model (66.7%); the highest specificity (72.5%) and PPV (33.3%) was found for the in-house *Borrelia* ELISpot assay when both antigens with their interaction term were included in the BLR model. The NPV was highest for the in-house *Borrelia* ELISpot assay, irrespective of whether or not the interaction term was included (85.7% each) (Table 3).

In conclusion, the two ELISpot assays showed a poor diagnostic performance for the diagnosis of active LNB when the numbers of SFCs were used in the ROC curve analyses, with sensitivities ranging from 44.4 to 66.7%, specificities from 42.0 to 72.5%, PPVs from 21.8 to 33.3% and NPVs from 80.5 to 87.0% (Table 3).

THE DIAGNOSTIC PERFORMANCE OF THE LYMESPOT ASSAY BASED ON THE SIS

ROC curve analysis based on a combination of the SIs of both antigens following the manufacturer's protocol (Supporting information, Fig. S2) resulted in an AUC of 0.487 (Table 3), which almost perfectly fitted the random predictor (Fig. 2d). Based on this analysis, the LymeSpot assay had a sensitivity of 27.8% to diagnose active LNB (Table 3). Thus, only five of the 18 active LNB patients were correctly identified using the LymeSpot assay (Table 4). Of these five active LNB patients, two had a positive LymeSpot result, and for three active LNB patients the LymeSpot results would still need diagnostic verification according to the manufacturer's instructions. The remaining 13 (72.2%) active LNB patients had a negative LymeSpot result (Table 4). When the results that needed diagnostic verification were excluded from the positive results, the sensitivity of the LymeSpot assay decreased to 11.1%. The specificity of the LymeSpot assay was 69.9% (Table 3), and 21 (30.4%) of the 69 controls either needed diagnostic verification [*n* = 11 (15.9%)] or had a positive LymeSpot result [*n* = 10 (14.5%)] (Table 4). Interestingly, the percentage of positive LymeSpot results was highest among treated healthy individuals [seven of 10 (70.0%)]; however, this was not significantly higher when compared to the other groups (adjusted *P* values > 0.025) (Table 4).

Table 4. Interpretation of the LymeSpot assay based on the stimulation indices according to the protocol of the manufacturer

			Statistics	
Study groups	**NEG (*n*; %)**	**POS[a] (*n*; %)**	**BH[b] (overall)**	**BH[b] (2-group)**
Active LNB patients (*n* = 18)	13 (72.2)	5[c] (27.8)	0.066	> 0.025[e]
Treated LNB patients (*n* = 12)	7 (58.3)	5[d] (41.7)		
Treated healthy individuals (*n* = 10)	3 (30.0)	7[f] (70.0)		
Untreated healthy individuals (*n* = 47)	38 (80.9)	9[g] (19.1)		
Control group[h] (*n* = 69)	48 (69.6)	21[i] (30.4)	NA	NA

NEG, negative; POS, positive; *n*, number of study participants; BH, Benjamini–Hochberg; LNB, Lyme neuroborreliosis;
NA, not applicable.
a. The positive results include those results that needed diagnostic verification.
b. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
c. Three (16.7%) of 18 active Lyme neuroborreliosis (LNB) patients required diagnostic verification.
d. Three (25.0%) of 12 treated LNB patients required diagnostic verification.
e. As the initial comparison was significantly different (raw *P* value < 0.050), two-group comparisons were also performed.
f. Two (20.0%) of 10 treated healthy individuals required diagnostic verification.
g. Six (12.8%) of 47 untreated healthy individuals required diagnostic verification.
h. The control group consists of all study participants except the active LNB patients.
i. 11 (15.9%) out of 69 controls required diagnostic verification.

ELISPOT RESULTS VERSUS CLINICAL SYMPTOMS, ANTIBIOTIC THERAPY AND RECOVERY STATUS

In total, 26 (29.9%) of the 87 study participants reported symptoms at inclusion in this study; all active LNB patients (*n* = 18) and eight (66.7%) of the 12 treated LNB patients. Overall, no association was found between the presence of symptoms and the results of the various ELISpot protocols (Table 5a). For treated LNB patients, the number of complaints reported at the start of the study was also not associated with the ELISpot results, irrespective of the ELISpot protocol used (Table 5a). As the treated healthy individuals were only included when they did not report any symptoms at the start of the study, elevated SFC counts in this group could not be linked to symptoms. Similarly, 16 (28.1%) of the 57 healthy individuals had a positive LymeSpot result, which could not be linked to symptoms.

Among active LNB patients, no association was found between the duration of symptoms prior to the blood sampling and the ELISpot results, irrespective of the ELISpot protocol used (Table 5b). For most active LNB patients, the antibiotic treatment had already started at the time of blood sampling; however, no association was found between the duration of antibiotic therapy prior to blood sampling and the ELISpot results using the various ELISpot protocols (Table 5c). Similarly, no association was found between the degree of recovery and the T-cell response of active LNB patients (Table 5d). For treated LNB patients, the degree of recovery was assessed at a median of 37.0 days after the end of antibiotic treatment for active disease in the past (approximately 5.4 years ago; Table 1), therefore, we did not compare the degree of recovery with the various ELISpot results obtained in the current study.

ELISPOT RESULTS VERSUS *BORRELIA*-SPECIFIC ANTIBODIES

In our previous study, elevated numbers of *B. burgdorferi* B31-specific T cells were significantly associated with the presence of *Borrelia*-specific serum antibodies [20]. In this study, which included a smaller number of study participants, comparison of the *B. burgdorferi* B31-specific SFC counts with the serology results showed a trend towards a combined B- and T-cell response when all study participants were analyzed together, irrespective of the ELISpot protocol used (Table 6a).

Within-group comparisons also showed a (non-significant) trend towards a combined B- and T-cell response, except for treated LNB patients, who showed elevated *B. burgdorferi* B31-specific SFC counts in the absence of *Borrelia*-specific antibodies. This was, again, in line with the results found in our previous study [20]. The presence of *Borrelia*-specific IgM or IgG also was not associated with elevated SFC counts (adjusted *P* values ranged from 0.199 to 1.000; data not shown).

Among active LNB patients, no significant association was found between the intrathecal production of *Borrelia*-specific antibodies and the T-cell response (Table 5b), which was similar to the results of our previous study in which 33 active LNB patients were included [20]. We also did not find a difference among the active LNB patients between negative and positive IgM AI results or between negative and positive IgG AI results when compared to the various SFC counts (adjusted *P* values ranged from 0.131 to 1.000; data not shown).

For treated LNB patients, the presence of intrathecally produced *Borrelia*-specific antibodies was determined at the time of active disease in the past, therefore, we did not compare these results with the ELISpot results using the various ELISpot protocols, as these were performed on average 5.4 years (Table 1), at the time the treated LNB patients were included in this study.

Table 5. Overview of the T-cell response, the presence and duration of clinical symptoms, and the degree of recovery (after antibiotic therapy) among the various study groups

				50 µl *B. burgdorferi* B31		100 µl *B. burgdorferi* B31			
				SFC count in-house *Borrelia* ELISpot assay		SFC count in-house *Borrelia* ELISpot assay		SFC count LymeSpot assay	
	Study groups	**Symptoms[a]**	***n* (%)**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**
(a)	All study participants (*n* = 87)	NO	61 (70.1)	2.0 (0.5-6.0)	0.439	3.0 (2.0-8.0)	0.486	4.5 (1.5-10.5)	0.916
		YES[c]	26 (29.9)	4.5 (1.3-7.4)		7.5 (1.5-13.9)		5.8 (2.1-9.6)	
	Treated LNB patients (*n* = 12)	NO	4 (33.3)	15.2 (7.9-24.2)	0.157	15.0 (5.0-25.4)	0.717	30.5 (9.9-54.1)	0.100
		YES[c]	8 (66.7)	2.0 (1.8-5.6)		9.5 (4.0-15.6)		3.3 (1.7-8.3)	
(b)	Symptom duration[d] (median; IQR)		*n* (%)	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]
	Active LNB patients	33.5 (15.8-59.5)	18 (100)	r_s = -0.200	0.703	r_s = -0.130	0.815	r_s = 0.170	0.746
(c)	Post AB-treatment time[e] (median; IQR)		*n* (%)	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]
	Active LNB patients	6.0 (3.3-7.0)	18 (100)	r_s = -0.160	0.770	r_s = -0.130	0.816	r_s = -0.370	0.374
(d)	Recovery status[f]		*n* (%)	Median (IQR)	BH[b]	Median (IQR)	BH[b]	Median (IQR)	BH[b]
	Active LNB	Incomplete	5 (27.8)	6.0 (2.0-9.5)	0.938	9.0 (5.0-14.5)	0.735	6.3 (1.0-10.0)	0.735
	patients (*n* = 18)	Complete	13 (72.2)	5.0 (1.0-7.0)		5.0 (1.0-10.0)		6.0 (3.0-11.0)	

Osp, outer surface protein; SFC, spot-forming cell; ELISpot, enzyme-linked immunosorbent spot; *n*, number of study participants; IQR, interquartile range; BH, Benjamini–Hochberg; NEG, negative; POS, positive; LNB, Lyme neuroborreliosis.

a. Symptoms are defined as the presence of symptoms at the start of the study. For (un)treated healthy individuals and treated Lyme neuroborreliosis (LNB) patients, the presence of symptoms was assessed by the completion of a Lyme-specific questionnaire; (un)treated healthy individuals were only included if they did not report any symptoms at the start of the study. For active LNB patients, the presence of symptoms was extracted from the hospital information system.
b. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).
c. In total eight (66.7%) of the 12 treated LNB patients reported complaints at the start of the study. For all treated LNB patients, the presence of complaints was reported on average 5.4 years after the diagnosis of active disease in the past (Table 1).
d. Symptom duration is defined as the number of days the study participant experienced complaints prior to blood sampling.
e. Post AB-treatment time is defined as the number of days between the start of antibiotic (AB) treatment and blood sampling (median days; IQR).
f. The degree of recovery (recovery status) was assessed after a median of 38.0 days after the end of antibiotic therapy for active disease (Table 1).

50 µl Osp-mix		**100 µl Osp-mix**						
SFC count in-house *Borrelia* ELISpot assay		***SFC count in-house* *Borrelia* ELISpot assay**		**SFC count LymeSpot assay**		**LymeSpot assay**		
Median (IQR)	**BH[b]**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**	**NEG**	**POS**	**BH[b]**
1.0 (0.0-2.0)	0.994	1.0 (0.0-3.0)	0.811	1.5 (0.5-3.5)	0.720	42	19	1.000
0.0 (0.0-4.8)		1.0 (0.0-3.0)		1.3 (0.3-2.5)		19	7	
2.0 (0.0-6.3)	1.000	1.0 (0.0-2.5)	1.000	2.8 (1.4-8.4)	0.570	1	3	0.497
0.5 (0.0-3.0)		0.5 (0.0-4.0)		1.3 (0.4-2.3)		6	2	
Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	LymeSpot assay	Symptom duration[d] (median; IQR)	BH[b]
r_s = 0.180	0.721	r_s = 0.180	0.722	r_s = 0.440	0.250	NEG (*n*=13) POS (*n*=5)	32 (14.0-45.0) 55 (22.0-204.0)	0.677
Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	Correlation coefficient	BH[b]	LymeSpot assay	Post AB-treatment time[e] (median; IQR)	BH[b]
r_s = -0.088	0.910	r_s = -0.098	0.889	r_s = -0.210	0.683	NEG (*n*=13) POS (*n*=5)	6.0 (5.0-8.0) 2.0 (2.0-6.0)	0.358
Median (IQR)	BH[b]	Median (IQR)	BH[b]	Median (IQR)	BH[b]	NEG	POS	BH[b]
0.0 (0.0-0.0)	0.460	1.0 (1.0-2.0)	0.992	0.5 (0.0-2.5)	0.784	4	1	1.000
1.0 (0.0-6.0)		0.0 (0.0-5.0)		1.5 (0.5-2.5)		9	4	

Table 6. Overview of the B- and T-cell response among the various study groups

				50 µl *Bb* B31		100 µl *Bb* B31	
				SFC count in-house *Borrelia* ELISpot assay		SFC count in-house *Borrelia* ELISpot assay	
	Study groups	**Serology result (IgM + IgG)**	***n* (%)**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**
(a)	All study participants combined (*n* = 87)	NEG	60 (69.0)	2.0 (0.4-5.3)	0.181	3.0 (1.8-6.0)	0.162
		POS	27 (31.0)	5.0 (1.5-8.8)		8.0 (3.0-14.5)	
	Active LNB patients (*n* = 18)	NEG	3 (16.7)	5.0 (2.5-19.5)	1.000	5.0 (2.5-28.0)	1.000
		POS	15 (83.3)	6.0 (1.5-7.5)		8.0 (2.0-11.0)	
	Treated LNB patients (*n* = 12)	NEG	11 (91.7)	6.0 (2.0-11.2)	0.783	7.0 (5.0-19.0)	1.000
		POS	1 (8.3)	2.0		12.0	
	Treated healthy individuals (*n* = 10)	NEG	6 (60.0)	4.5 (1.5-10.9)	0.359	3.5 (3.0-4.8)	0.130
		POS	4 (40.0)	24.0 (12.5-33.5)		45.0 (19.2-75.2)	
	Untreated healthy individuals (*n* = 47)	NEG	40 (85.1)	1.3 (0.0-3.0)	0.551	2.0 (1.0-4.3)	0.581
		POS	7 (14.9)	3.0 (0.8-5.5)		6.0 (2.0-11.2)	
		AI result (IgM + IgG)					
(b)	Active LNB patients	NEG	6 (33.3)	3.5 (2.3-19.0)	1.000	4.0 (1.5-26.4)	1.000
		POS	12 (66.7)	6.0 (0.8-7.3)		8.0 (2.3-10.5)	

Bb B31, *B. burgdorferi* B31; Osp, outer surface protein; SFC, spot-forming cell; ELISpot, enzyme-linked immunosorbent spot; AI, antibody index; *n*, number of study participants; IQR, interquartile range; BH, Benjamini–Hochberg; NEG, negative; POS, positive; LNB, Lyme neuroborreliosis; AB, antibiotic treatment for Lyme borreliosis.

a. The final LymeSpot result is based on a combination of the stimulation indices of both antigens following the protocol of the manufacturer (Supporting information, Fig. S2).

b. To correct for multiple comparisons, the Benjamini–Hochberg procedure was applied with a false discovery rate of 2.5% (adjusted *P* values are shown).

		50 µl Osp-mix		100 µl Osp-mix						
SFC count LymeSpot assay		**SFC count in-house *Borrelia* ELISpot assay**		**SFC count in-house *Borrelia* ELISpot assay**		**SFC count LymeSpot assay**		**Final LymeSpot result[a]**		
Median (IQR)	**BH[b]**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**	**Median (IQR)**	**BH[b]**	**NEG**	**POS**	**BH[b]**
3.4 (1.4-8.6)	0.107	1.0 (0.0-2.0)	1.000	1.0 (0.0-3.0)	0.769	1.0 (0.4-3.0)	0.672	43	17	0.986
7.0 (3.8-19.4)		0.0 (0.0-4.5)		1.0 (0.0-4.0)		2.0 (0.5-4.0)		18	9	
11.0 (6.3-30.2)	0.685	1.0 (0.5-5.0)	0.630	5.0 (2.5-6.0)	0.652	4.5 (2.3-8.3)	0.691	1	2	0.430
6.0 (2.8-9.3)		0.0 (0.0-4.5)		1.0 (0.0-2.5)		1.0 (0.3-2.5)		12	3	
8.3 (2.3-15.2)	1.000	1.0 (0.0-5.0)	0.355	0.0 (0.0-3.0)	1.000	1.5 (0.8-3.5)	1.000	6	5	1.000
8.5		0.0		1.0		1.5		1	0	
8.5 (2.4-15.8)	0.111	1.8 (0.4-2.0)	0.724	2.0 (0.5-2.8)	0.418	3.8 (2.8-6.2)	0.309	3	3	0.471
78.8 (66.8-83.1)		9.5 (5.5-14.2)		11.0 (6.8-13.6)		12.2 (10.0-12.6)		0	4	
3.0 (1.2-5.6)	0.377	1.0 (0.0-1.3)	0.785	1.0 (0.0-2.0)	0.679	1.0 (0.0-2.1)	0.469	33	7	0.814
5.0 (3.5-10.0)		0.0 (0.0-1.0)		2.0 (0.5-4.0)		2.5 (1.0-3.8)		5	2	
4.3 (2.6-8.9)	0.847	2.0 (0.0-5.5)	0.845	0.5 (0.0-1.8)	0.890	0.3 (0.0-2.0)	0.625	4	2	1.000
6.6 (4.6-10.4)		0.0 (0.0-2.8)		1.0 (0.0-3.5)		1.5 (0.5-2.9)		9	3	

DISCUSSION

In the current study, the diagnostic performance of two ELISpot assays to diagnose active LNB were compared. The final study population consisted of 87 participants and comprised 18 active and 12 treated LNB patients, 10 healthy individuals who were treated for an early (mainly cutaneous) manifestation of LB in the past and 47 untreated healthy individuals. Both our in-house *Borrelia* ELISpot assay and the LymeSpot assay showed a poor diagnostic performance based on the numbers of SFCs with AUCs ranging from 0.429 to 0.570. The corresponding sensitivities, specificities, PPVs and NPVs ranged from 44.4 to 66.7%, 42.0 to 72.5%, 21.8 to 33.3% and 80.5 to 87.0%, respectively. The diagnostic performance of the LymeSpot assay, using so-called SIs following the manufacturer's protocol, resulted in a comparably low AUC of 0.487, with a corresponding sensitivity of 27.8%, a specificity of 69.6%, a PPV of 19.1% and a NPV of 78.6%. Our study showed that the two ELISpot assays, irrespective of the protocol used, cannot be used to diagnose LNB or to monitor antibiotic treatment success.

The results of the 87 study participants of the in-house *Borrelia* ELISpot assay after stimulation of the PBMCs with 50 µl of *B. burgdorferi* B31 in the current study represent a subset of the results of the 243 study participants published previously [20]. The SFC counts between the four study groups of the subgroup in this study were comparable with the SFC counts between the four study groups of the entire study population. Both studies showed significantly higher numbers of SFCs after stimulation with *B. burgdorferi* B31 for treated healthy individuals compared to untreated healthy individuals. Active LNB patients and treated LNB patients also showed higher numbers of SFCs after stimulation with *B. burgdorferi* B31 compared to untreated healthy individuals, although not significant in the current study. This is most probably explained by the lower number of study participants per group in the current study. The association between the B- and T-cell response that was found in our previous study was also seen in the current study, although it was not significant, most probably due to the smaller study population. The overall conclusion, that elevated numbers of SFCs are associated with a previous contact with the *Borrelia* bacterium [20]; however, was confirmed and could not be linked to symptomology nor to the degree of recovery or to antibiotic treatment. Elevated IFN-γ levels among asymptomatic individuals and previous LB patients have also been found by others [32-35].

Comparison of the in-house *Borrelia* ELISpot results after PBMC stimulation with either 50 or 100 µl of *B. burgdorferi* B31 showed similar results for three of the four study groups. However, among untreated healthy individuals, significantly higher numbers of SFCs were seen when 100 µl was used. This could be explained by the relatively higher number of untreated healthy individuals compared to the number of study participants in the other three groups.

Consistent with our previous study [20], we found that the use of Osp-mix as a T-cell stimulant resulted in very low numbers of SFCs, and cannot be used in its current composition to distinguish active LNB patients from the three control groups. Other studies also described a reduced performance of recombinant antigens compared to whole-cell lysates [13, 36]. This may, in part, be explained by the number of different antigens present: (a mixture of various) recombinant antigens contains far less antigens than a whole-cell lysate. Alternatively, recombinant antigens are more specific, therefore limiting the possibility of cross-reactivity. It is known that *Borrelia*-specific antibodies show cross-reactivity with other diseases [37] and that the bacterium shows high sequence homology with bacteria such as *Treponema* or *Leptospira* [38, 39]. Cross-reactivity could theoretically result in higher numbers of SFCs when a whole-cell lysate of *B. burgdorferi* B31 is used in patients with an active or previous infection caused by bacteria such as *Treponema* or *Leptospira*, or in healthy individuals who carry non-pathogenic *Treponema* or *Leptospira* species. Previously, we have tested two patients with active leptospirosis in our in-house *Borrelia* ELISpot assay, and one of them had high numbers of SFCs after stimulation with a whole-cell lysate of *B.*

burgdorferi B31 [20].

Overall, the numbers of SFCs after stimulation with *B. burgdorferi* B31 were also relatively low. In our experience, as well as described by others - for tuberculosis or cytomegalovirus infections - the numbers of IFN-γ-secreting T cells among exposed or infected individuals measured in an ELISpot assay using comparable amounts of PBMCs, ranging from 2.0×10^5 to 2.5×10^5, are generally much higher [40-42]. The lack of T-cell activity among the active LNB patients could be explained by the choice of *Borrelia* antigens. In the Netherlands, LNB is mainly caused by *B. garinii* and *B. bavariensis* [43] and less frequently by *B. burgdorferi* sensu stricto. As we have discussed previously [20], we do not believe that the use of *B. burgdorferi* B31 whole-cell lysate in the ELISpot assay resulted in the poor performance of both ELISpot assays, as *B. burgdorferi*, *B. garinii* and *B. bavariensis* are closely related and share many antigens. Von Baehr et al. [13] evaluated three *Borrelia* species and did not find any difference. Nordberg et al. [44] used *B. garinii* as a stimulating agent, CSF instead of blood and nitrocellulose-bottomed ELISpot plates instead of PVDF-bottomed plates, and also did not find higher numbers of activated T cells in their ELISpot assay. The Osp-mix we used contained antigens derived from an LNB-associated strain (*B. garinii*); however, the Osp-mix was inferior compared to the use of *B. burgdorferi* B31, as discussed in the previous paragraph. The lack of T-cell activity might also be explained by the inability of the human host to develop an adequate immune response against the *Borrelia* bacterium or the ability of the *Borrelia* bacterium to escape or suppress the immune system [45, 46]. It could also be due to the disease manifestation that was studied, as already debated previously [20], as LNB implies a local infection of the brain. Testing blood might thus be less suitable, as the immune cells could have migrated towards the central and/or peripheral nervous system [47, 48]. The testing of CSF, in combination with blood, may be more suitable [49]. Furthermore, IFN-γ may not the best marker to diagnose active LNB. It would be interesting to investigate whether other cytokines and/or chemokines could improve the ELISpot assays tested in this study. Recently, the LymeSpot assay has been adapted by the manufacturer by adding the detection of interleukin (IL)-2. However, no data are available yet with regard to the diagnostic performance of this modified LymeSpot assay.

For the LymeSpot assay, the PBMC isolation procedure used in this study deviated from the manufacturer's (AID) recommended protocol. These deviations from the LymeSpot protocol were made in order for the technician to be able to perform and process the ELISpot assays simultaneously, and to minimize the differences between the assays to allow for a more fair comparison. The PBMCs used in the LymeSpot assay were thus isolated according to the same protocol that was already in use in our laboratory for the in-house *Borrelia* ELISpot assay [20] and for the T-SPOT.TB test [20, 26, 50]. Consequently, the PBMC isolation differed at four points compared to the instruction manual of the LymeSpot assay.

First, the medium to dilute the blood prior to PBMC isolation differed, as RPMI medium was used instead of phosphate-buffered saline (PBS). Secondly, Leucosep tubes were used for the isolation of PBMCs, while the LymeSpot protocol advises to use standard tubes with a Ficoll gradient. As a consequence, the centrifugation steps of the isolation procedure were adjusted based on the instruction manual supplied with the Leucosep tubes. As the isolation of PBMCs is based on a gradient, we do not believe that the altered centrifugation time resulted in a different PBMC yield. An increased centrifugation speed could, potentially, result in a higher PBMC yield, but this should not influence the results of the LymeSpot assay, as the amount of PBMCs per well is standardized. This is confirmed by others [51, 52], who showed that PBMCs isolated by Leucosep tubes performed equally well in the ELISpot assay compared to PBMCs isolated using the Ficoll-gradient technique.

Thirdly, the centrifugation steps that were used to wash the PBMCs and the number of times the PBMCs were washed differed from the LymeSpot protocol. However, in the literature, various centrifugation speeds and times for washing the PBMCs are described, which range from 300 to

640 *g* for 7-10 min for the first wash step and from 300 to 470 *g* for 7-10 min for the second wash step [26, 41, 51, 53-55].

Finally, the amount of PBMCs used varied slightly, as we used 2.5×10^5 PBMCs/well, and according to the LymeSpot assay, 2.0×10^5 PBMCs/well should have been used. A higher number of PBMCs per well could result in increased numbers of SFCs, as the use of more PBMCs results in more antigen-presenting cells and more T cells that could become activated after stimulation with the *Borrelia* antigens.

The results of a comparative pilot experiment that we performed in which we assessed the influence of the deviations discussed above supported that these deviations from the recommended protocol are not critical as such (Supporting information, Data S4). Hence, the conclusion stands that both ELISpot assays cannot help to diagnose active LNB.

Probably some of the most critical steps that influence the performance of an ELISpot assay are the time between venipuncture and PBMC isolation, the time between PBMC isolation and incubation of the assay and the (overnight) incubation time of the assay [56, 57]. In this study, these times were all within the limits as described in the LymeSpot protocol, with the exception of the time between venipuncture and PBMC isolation, which was prolonged for various cases. A prolonged time between venipuncture and PBMC isolation is known to decrease the PBMC viability [56]. To compensate for this, for those cases for which the time between venipuncture and PBMC isolation was prolonged (8–32 h), we performed a T-Cell Xtend step prior to PBMC isolation. This T-Cell Xtend step has proved not to be detrimental to the PBMC yield and the ELISpot performance [26, 27, 58].

No data are provided in the instruction manual of the LymeSpot assay with regard to the diagnostic performance of this assay. To our knowledge, this is the first study that has investigated the diagnostic capacity of the LymeSpot assay for the diagnosis of active LNB. The diagnostic performance of the LymeSpot assay for other manifestations of LB has not yet been investigated thoroughly and remains unclear. Hopefully, more validation studies will be performed which will include other manifestations of LB, as well as follow-up studies to understand more clearly the diagnostic potential for treatment monitoring.

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DISCLOSURES

None.

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SUPPLEMENTAL MATERIAL

Table S1. Comparison of the protocols used for the evaluation of the in-house *Borrelia* ELISPOT assay and the LymeSpot assay

Steps in the ELISPOT assay procedure	Protocol	
	In-house *Borrelia* ELISPOT assay	**LymeSpot assay**
ELISPOT plate	96-wells PVDF plate coated with anti-human IFN-γ antibodies (supplied by Mabtech)	96-wells PVDF plate coated with anti-human IFN-γ antibodies (supplied by AID)
PMBC isolation	In-house *Borrelia* ELISpot protocol as described in the "Materials and Methods" section of the published manuscript [1]	*Altered protocol*[a]: identical to the in-house *Borrelia* ELISPOT assay
PMBC concentration	100 µl of 2.5 x 10^6 PBMCs/ml	*Altered protocol*[a]: identical to the in-house *Borrelia* ELISPOT assay
Negative control	50 µl of AIM-V medium (supplied by Life technologies); tested in the singular	100 µl of AIM-V medium (supplied by Life technologies); tested in duplicate
Positive control	50 µl (0.1 µg/ml) of anti-human CD3 MAb CD3-2 (supplied by Mabtech); tested in the singular	100 µl of Pokeweed (supplied by AID); tested in duplicate
Borrelia antigens	Both 50 µl and 100 µl of *B. burgdorferi* B31 whole- cell lysate[b] (5 µg/ml) (supplied by AID); both tested in the singular	100 µl of *B. burgdorferi* B31 whole-cell lysate (5 µg/ml) (supplied by AID); tested in duplicate
	Both 50 µl and 100 µl of Osp-mix[c] (5 µg/ml) (supplied by AID); both tested in the singular	100 µl of Osp-mix[c] (5 µg/ml) (supplied by AID); tested in duplicate
Incubation time	20-24 hours at 37°C and 5% CO2	Identical to the in-house *Borrelia* ELISPOT assay
Wash step	Four times with an excess of PBS (pH 7.2 ± 0.1)	Six times 200 µl of washing buffer 'WP' (supplied by AID)
Conjugate	50 µl of 7-B6-1–alkaline phosphatase conjugated secondary antibody (supplied by Mabtech)	100 µl of alkaline phosphatase conjugated secondary antibody (supplied by AID)
Incubation time	One hour at 2°C	Two hours at room temperature in a humidified chamber
Wash step	Four times with an excess of PBS (pH 7.2 ± 0.1)	Six times with 200 µl of washing buffer 'WP' (supplied by AID)
Substrate	50 µl of BCIP/NBT plus substrate (supplied by Mabtech)	100 µl of BCIP/NBT substrate (supplied by AID)
Incubation time	7-10 minutes at room temperature or until SFCs become clearly visible	5-20 minutes at room temperature or until SFCs become clearly visible
Wash step	Four times with excess of tap water	Three times with excess of tap water
Dry plate	At least 90 minutes at 37°C	Identical to the in-house *Borrelia* ELISPOT assay
Calculation method of the final results of both ELISPOT assays	• Positive control should have ≥20 SFCs • Negative control should have ≤6 SFCs • 50 µl of *Borrelia* antigen: subtract no. of SFCs in the negative control well from those in the *Borrelia* antigen-stimulated well • 100 µl of *Borrelia* antigen: multiply no. of SFCs in the negative control well by two and subtract from no. of SFCs in the *Borrelia* antigen-stimulated well	Two calculation methods were performed: 1. A calculation based on the protocol of the in-house *Borrelia* ELISPOT assay, for which the no. of SFCs in the negative control were subtracted from the no. of SFCs in the *Borrelia* antigen-stimulated well (take average for all duplicates); 2. A calculation based on the protocol of the manufacturer (AID) as is shown in Fig. S2.

PVDF, polyvinylidene difluoride; IFN-γ, interferon-gamma; AID, Autoimmun Diagnostika GmbH; PBMC, peripheral blood mononuclear cell; CD, cluster of differentiation; MAb, monoclonal antibody; PBS, phosphate-buffered saline; BCIP/NBT, 5-bromo-4-chloro-3'-indolylphosphate and nitroblue tetrazolium; SFCs, spot-forming cells (SFCs are based on the numbers of IFN-γ-secreting T cells/ 2.5 x 10^5 PBMCs).

a. The altered protocol showed various deviations from the LymeSpot protocol, which are described in the "Discussion" section of the published manuscript [1]. The influence of these deviations on the diagnostic performance of the LymeSpot assay are assessed in Data S4.

b. For some cases who were tested in the in-house *Borrelia* ELISPOT assay, more than one lot number of *B. burgdorferi* B31 whole-cell lysate (50 µl protocol) was used to stimulate the PBMCs. For these cases, the median number of SFCs was used for all subsequent analyses.

c. The outer surface protein (Osp)-mix consisted of a pool of 9-mer to 11-mer peptides of Osp-A (*B. burgdorferi, B. afzelii*, and *B. garinii*), native Osp-C (*B. afzelii*), and recombinant p18.

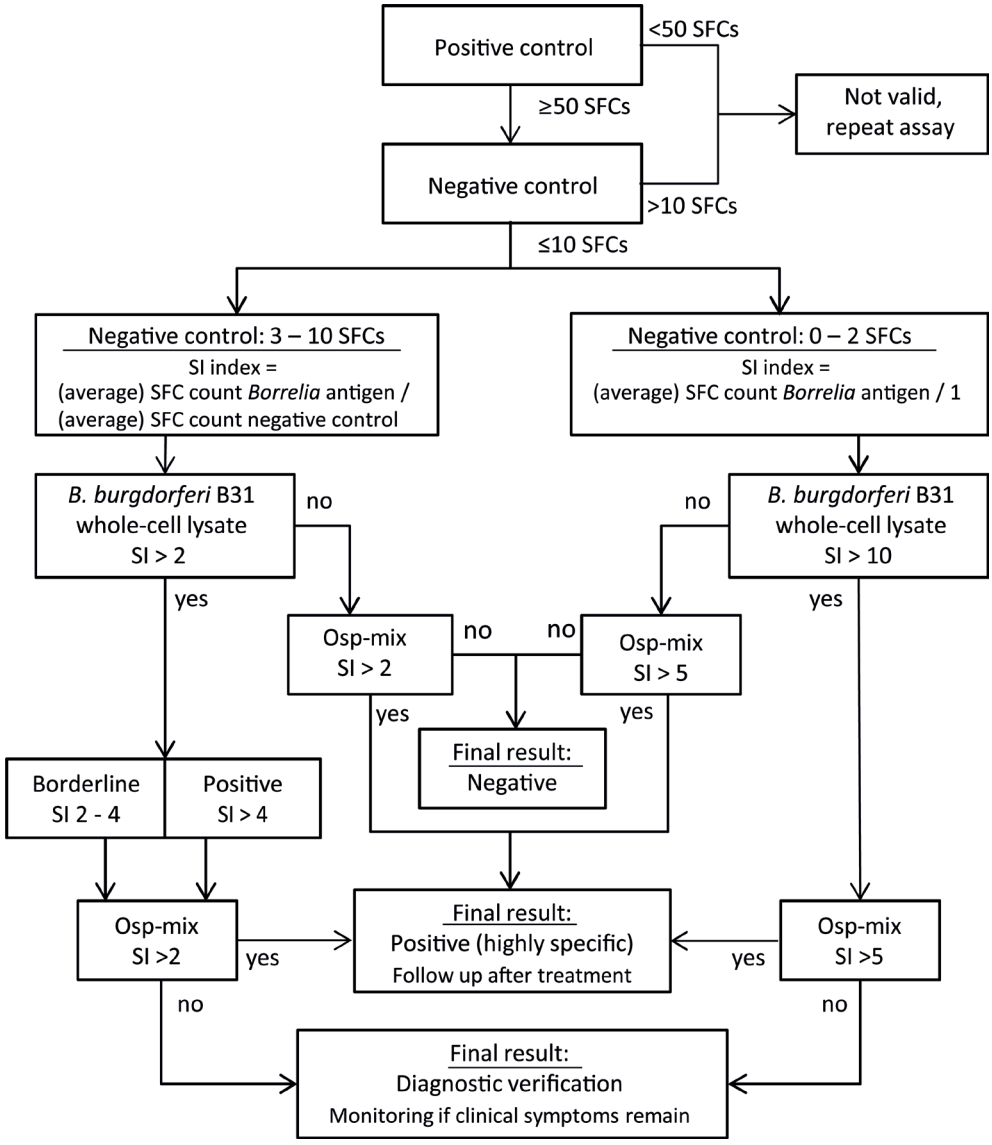

Fig. S2. Interpretation scheme of the LymeSpot assay according to the manufacturer. The spot-forming cell (SFC) count is based on the numbers of interferon-gamma-secreting T cells/2.5 x 10^5 PBMCs upon stimulation with Pokeweed (positive control), AIM-V medium (negative control), *B. burgdorferi B31* whole-cell lysate, or a mixture of various recombinant outer surface proteins (Osp). The Osp-mix consists of a pool of 9-mer to 11-mer peptides of Osp-A (*B. burgdorferi*, *B. afzelii*, and *B. garinii*), native Osp-C (*B. afzelii*), and recombinant p18. Depending on the numbers of SFCs in the negative control well, a stimulation index (SI) is calculated.

Table S3. Overview of the study populations used in the three studies

Studies		Time of inclusion of the study participants[a]																																					
		2010				2011				2012				2013				2014				2015				2016				2017				2018							
Reference	Study participants	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
	Total number of active Lyme NB patients (*n* = 40)					2		2	2			3		1	1	2	1	1	1	3	3	2	1	1	3	1		4	3			1	1	1					
2	*n* = 27					2		2	2			3		1	2	1	1	1	3	2		2	1	1	3														
3	*n* = 33					2		2	1			3		1	1	2	1	1	1	2	3		1	1	3	1		4	3										
1	*n* = 18																	1	1	1	2			1	2			4	3			1	1	1					
	Total number of treated Lyme NB patients (*n* = 39)					1								11				15				12																	
2	*n* = 36													10				15				11																	
3	n = 37					1								11				13				12																	
1	n = 12																					12																	
	Total number of treated healthy individuals (*n* = 28)													14				4				1	3		6														
2	*n* = 27													13				4				1	3		6														
3	*n* = 28													14				4				1	3		6														
1	*n* = 10																	2				3			5														
	Total number of untreated healthy individuals (*n* = 148)													69				64				10	2		3														
2	*n* = 147													69				63				10	2		3														
3	*n* = 145													67				64				9	2		3														
1	*n* = 47																	35				9	1		2														

Q1, first quarter; Q2, second quarter; Q3, third quarter; Q4, fourth quarter; LNB, Lyme neuroborreliosis

a. The number of study participants used in the three studies are in principle overlapping, unless for instance serum [2], or the number of peripheral blood mononuclear cells ([3], or current study [1]) were insufficient. The underlined numbers reflect the number of study participants (n=84) used in both ELISpot studies ([3] and current study [1]).

Data S4. A pilot study to investigate the influence of three different protocols for the isolation of peripheral blood mononuclear cells (PBMCs) on the LymeSpot results

INTRODUCTION OF DATA S4

PBMCs are immune cells that can be used to investigate the cellular immune response during an infection. To isolate PBMCs from whole blood, density gradient centrifugation can be applied followed by several wash steps to remove platelets, erythrocytes, granulocytes and residual Ficoll [4]. In the current manuscript of Van Gorkom et al. [1], we validated the LymeSpot assay. At the same time, the numbers of spot-forming cells (SFCs) obtained by using the LymeSpot assay were compared with those obtained by using our in-house *Borrelia* enzyme-linked immunosorbent spot (ELISpot) assay. To minimize the differences between both ELISpot assays and to facilitate the technician who performed both assays simultaneously, we used one single PBMC isolation protocol: the one that was already in use in our laboratory for the in-house *Borrelia* ELISpot assay. Consequently, the PBMC isolation protocol we used for the LymeSpot assay deviated from the protocol recommended by the manufacturer (Autoimmun Diagnostika (AID) GmbH, Straßberg, Germany). Our in-house PBMC isolation protocol differed from the LymeSpot protocol on four points: (i) the PBMC separation medium used, (ii) the various centrifugation steps, (ii) the medium used to wash the PBMCs, and (iv) the amount of PBMCs tested per well (Table S4.1). In 2018, after our study had finished, the manufacturer of the LymeSpot assay implemented a number of changes to their recommended PBMC isolation protocol, most importantly the speed of the various centrifugation steps. Interestingly, in this 'new' LymeSpot protocol, the centrifugation speeds were increased compared to the previous 'old' LymeSpot protocol, and now more closely resembled the centrifugation speeds of our in-house *Borrelia* ELISpot protocol. As variations in the PBMC isolation protocols could potentially influence the final LymeSpot results, we conducted a pilot study to assess the influence of the various PMBC isolation protocols on the diagnostic performance of the LymeSpot assay. In this experiment we compared the LymeSpot results obtained by using our in-house PBMC isolation protocol with the LymeSpot results obtained by using the old and the new LymeSpot protocol.

Table S4.1. Overview of the three PBMC isolation protocols used in this pilot study

	Old LymeSpot assay [a]	New LymeSpot assay[b]	In-house *Borrelia* ELISPOT assay[c]
PBMC separation medium	Ficoll Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)	Identical to old LymeSpot protocol	Leucosep tubes (OxFord Immunotec Ltd., Abingdon, UK)
Centrifugation step	30 min at 400 *g* without break	30 min at 1000 *g* without break	15 min at 1000 *g* without break
First wash step	Isolate the PBMC layer and add the layer to a tube containing 10 ml of PBS (pH 7.4) (Fisher Scientific, Landsmeer, The Netherlands)	Identical to old LymeSpot protocol	Isolate the PBMC layer and add to a tube containing 10 ml of RPMI medium (Life Technologies, Invitrogen, Bleiswijk, The Netherlands)
Centrifugation step	Centrifuge 10 min at 350 *g* with break	Centrifuge 10 min at 700 *g* with break	Centrifuge 7 min at 600 *g* with break
Second wash step	Discard PBS, resuspend PBMCs and fill with 10 ml of PBS	Identical to old LymeSpot protocol	Discard RPMI, resuspend PBMCs and fill with 10 ml of RPMI medium (Life Technologies)
Centrifugation step	Centrifuge 10 min at 350 *g* with break	Centrifuge 10 min at 700 *g* with break	Centrifuge 7min at 300 *g* with break
Third wash step	Discard PBS, resuspend PBMCs in 10 ml of RPMI medium	Discard PBS, resuspend PBMCs in 10ml of AIM-V medium (Life Technologies)	No third wash step
Centrifugation step	Centrifuge 10 min at 350 *g* with break	Centrifuge 10 min at 700 *g* with break	No third centrifugation step
Count PBMC suspension	Discard medium, resuspend pellet in 1.1 ml AIM-V medium (supplied by Life technologies) and count PBMCs as described in the 'Materials and Methods' section of the current manuscript of van Gorkom et al. [1]	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
Adjust PBMC suspension	Adjust PBMC suspension to 2.0 x 10^6 cells/ml with AIM-V medium	Identical to old LymeSpot protocol	Adjust PBMC suspension to 2.5 x 10^6 cells/ml with AIM-V medium
Volume of PBMC suspension/well	Add 100 µl of PBMC suspension in each well of the LymeSpot plate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
For each subject the following wells were tested:			
Negative control	• 100 µl of AIM-V medium; tested in duplicate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
Positive control	• 100 µl of Pokeweed (supplied by Autoimmun Diagnostika (AID) GmbH, Straßberg, Germany); tested in duplicate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
Borrelia antigens	• 100 µl of *B. burgdorferi* B31 whole- cell lysate (5 µg/ml) (supplied by AID); tested in duplicate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol
	• 100 µl of Osp-mix[c] (5 µg/ml) (supplied by AID); tested in duplicate	Identical to old LymeSpot protocol	Identical to old LymeSpot protocol

PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; RPMI, Roswell Park Memorial Institute; Osp, outer surface protein
a. This protocol was valid from 2013 till 2018.
b. This protocol was made effective in 2018.
c. This protocol has been used in the current manuscript of Van Gorkom et al. [1].

MATERIALS AND METHODS OF DATA S4

SELECTION OF THE STUDY PARTICIPANTS

For this pilot study we recruited study participants, who represent a biased selection of the study participants described in the current manuscript of Van Gorkom et al. [1] and in the manuscript of Van Gorkom et al. published in 2018 [3]. These newly recruited study participants were a convenience sample of the healthy individuals. They were chosen because they were either working at the Diakonessenhuis Hospital or at the National Institute for Public Health and the Environment (RIVM) and, thus, were easily accessible, and because they previously had had positive ELISpot results, they had a good chance of yielding positive results again. Table S4.2 shows the characteristics of the seven participants included in this pilot study. Three study participants had previously been classified as untreated healthy individuals, and four had previously been classified as treated healthy individuals [1]. All study participants had previously been tested in the in-house *Borrelia* ELISpot assay and had shown elevated SFC counts and four of them had also been subjected to the LymeSpot assay and had tested positive. The remaining three study participants had not been tested in the LymeSpot assay previously, as they were initially recruited before we started the validation of the LymeSpot assay.

Table S4.2 Overview of the selected study participants for this pilot study and the results of previous ELISPOT assays

Study participant	Group	Year of first ELISPOT result	Median SFC count in-house *Borrelia* ELISPOT assay[a] using *B. burgdorferi* B31 whole-cell lysate (50µl)	LymeSpot results[a,b]
1	UHI	2013	16.3	ND
2	"	2013	8.5	ND
3	"	2015	20	POS
4	THI	2013	14	ND
5	"	2015	12.5	POS
6	"	2015	35	POS
7	"	2015	31	POS

UHI, untreated healthy individuals; THI, treated healthy individuals; SFC, spot forming cell; ND, not done; POS, positive

a. A total number of 2.5 x 10^5 peripheral blood mononuclear cells/well was used in the ELISPOT assay.
b. The LymeSpot result was based on a combination of both stimulation indices as described by the manufacturer (Fig. S2) of the current manuscript of Van Gorkom et al. [1].

PBMC ISOLATION AND LYMESPOT PROCEDURE

Table S4.1 summarizes the three protocols used for the isolation of the PBMCs in this pilot study. For each study participant, three lithium heparin tubes were collected. Separation of PBMCs was done by using density gradient centrifugation and for the old and new LymeSpot protocol we used Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). If isolation of PBMCs started within 8 hours after venipuncture, then PBMCs were isolated directly following the protocols described in Table S4.1. If isolation of PMBCs was done between 8 and 32 hours after venipuncture, then a T-Cell Xtend (OxFord Immunotec Ltd., Abingdon, United Kingdom) procedure was performed first, according to the protocol described in the 'Materials and Methods' of the current manuscript of Van Gorkom et al. [1]. The final LymeSpot result, as described in the instruction manual of the LymeSpot assay, was based on a combination of the stimulation indices of both antigens and was only calculated when the assay was valid (Fig. S2, and the 'Materials and Methods' section of the current manuscript of Van Gorkom et al. [1], respectively). To assess the differences between the SFC counts for the various isolation protocols, untreated and treated healthy individuals were analyzed separately, similar to the study population in the current manuscript of Van Gorkom et al. [1]. To determine the possible impact of the use of a

modified PBMC protocol for assessing the diagnostic performance of the LymeSpot assay, we classified final LymeSpot results that needed diagnostic verification as 'positive'. Subsequently, we combined those results with the positive results, as was done for the assessment of the diagnostic performance of the LymeSpot assay in the current manuscript of Van Gorkom et al. [1]

DATA HANDLING AND STATISTICAL ANALYSIS

The non-parametric Cochran's Q test for more than two related samples was used for comparison of the final LymeSpot results between the three protocols. Quantitative, related data comparing more than two groups were analyzed by using the Wilcoxon signed rank test with continuity correction. As all raw *P* values were >0.05, no correction was applied to account for the multiple statistical analyses in this pilot study.

RESULTS OF DATA S4

THE SFC COUNTS OBTAINED BY USING THE THREE PBMC ISOLATION PROTOCOLS

For five (71.4%) of the seven study participants, PBMCs were isolated within three hours of venipuncture, and for one untreated healthy individual (participant 1), and one treated healthy individual (participant 5), the venipuncture was performed on the previous day and therefore a pre incubation step with T-Cell Xtend was needed. Overall, all study participants that were included showed elevated SFC counts in at least one of the three protocols conducted, for at least one of the two *Borrelia* antigens tested (Table S4.3).

Comparison of the mean SFC counts among untreated healthy individuals and among treated healthy individuals did not show a difference for any of the protocols in case *B. burgdorferi* B31 whole-cell lysate was used to stimulate the PBMCs (raw *P* values 0.125 to 0.625) (Table S4.3). Similarly, no differences were found between the mean SFC counts for the three protocols when Osp-mix was used to stimulate the PBMCs (raw *P* values 0.125 to 1.000) (Table S4.3).

For both untreated and treated healthy individuals, more variation was seen between the mean SFC counts obtained by using the in-house *Borrelia* ELISpot protocol than between the mean SFC counts obtained by using the old and the new LymeSpot protocol (Table S4.3). The observed variation seemed to decrease when the mean SFC counts decreased. Comparison of the SFC counts between duplicate measurements also showed variation, which was largest for the in-house *Borrelia* ELISpot protocol when compared to the two LymeSpot protocols. The difference between the two duplicate measurements after stimulation of the PBMCs with *B. burgdorferi* B31 whole-cell lysate varied between 5 and 49 SFCs for the in-house *Borrelia* ELISpot protocol, between 1 and 27 SFCs for the new LymeSpot protocol, and between 0 and 8 SFCs for the old LymeSpot protocol (Table S4.3). The difference between the two duplicate measurements after stimulation of the PBMCs with Osp-mix varied between 0 and 11 SFCs for the in-house *Borrelia* ELISpot protocol, between 0 and 6 SFCs for the new LymeSpot protocol, and between 0 and 5 SFCs for the old LymeSpot protocol (Table S4.3).

Table S4.3. Results of the LymeSpot assay by using the three PBMC isolation protocols used in this pilot study

		Old LymeSpot protocol (protocol 1)			New LymeSpot protocol (protocol 2)			In-house *Borrelia* ELISPOT protocol (protocol 3)			Comparison of the three protocols based on			
		Mean SFC count (duplicate measurements)		Final LymeSpot result[a]	Mean SFC count (duplicate measurements)		Final LymeSpot result[a]	Mean SFC count (duplicate measurements)		Final LymeSpot result[a]	Mean SFC count			Final LymeSpot result[a]
Study participant	Group	*Bb* B31	Osp-mix		*Bb* B31	Osp-mix		*Bb* B31	Osp-mix		Protocols	Raw *P* values: *Bb* B31	Raw *P* values: Osp-mix	Raw *P* value
1[b]	Untreated healthy individuals	15 .0 (8.0-22.0)	ND[c]	DV/POS[c]	1.0 (0.0-2.0)	0.0 (0.0-0.0)	NEG	7.0 (4.0-10.0)	2.5 (1.0-4.0)	NEG	1 vs 2	0.250	1.000	0.223
2		11.5 (7.0-16.0)	2.0 (2.0-2.0)	DV	5.5 (4.0-7.0)	2.0 (0.0-4.0)	NEG	54.5 (43.0-66.0)	31.5 (31.0-32.0)	POS	1 vs 3	0.500	1.000	
3		28.5 (28.0-29.0)	9.0 (6.0-12.0)	POS	12.0 (12.0-12.0)	7.5 (5.0-10.0)	POS	41.5 (17.0-66.0)	6.5 (5.0-8.0)	DV	2 vs 3	0.250	0.500	
4	Treated healthy individuals	40.5 (27.0-54.0)	16.5 (15.0-18.0)	POS	11.5 (11.0-12.0)	6.0 (5.0-7.0)	POS	48.0 (42.0-54.0)	23.5 (18.0-29.0)	POS	1 vs 2	0.625	0.750	0.368
5[b]		6.0 (5.0-7.0)	4.5 (4.0-5.0)	NEG	12.5 (9.0-16.0)	4.0 (2.0-6.0)	DV	26.0 (23.0-29.0)	7.5 (5.0-10.0)	POS	1 vs 3	0.125	0.250	
6		25.5 (25.0-26.0)	ND[c]	DV/POS[c]	17.5 (16.0-19.0)	5.0 (3.0-7.0)	DV	54.5 (52.0-57.0)	10.0 (10.0-10.0)	POS	2 vs 3	0.125	0.125	
7		11.5 (11.0-12.0)	6.0[d]	POS	14.0 (10.0-18)	12.0 (11.0-13.0)	POS	53.0 (42.0-64.0)	27.5 (22.0-33.0)	POS				
Total no. of negative results for the LymeSpot assay				1 out of 7			2 out of 7			1 out of 7				
(no. out of total number of study participants (%))				14.3			28.6			14.3				

SFC, spot forming cell; *Bb* B31, *B. burgdorferi* B31 whole-cell lysate; Osp, outer surface protein, ND, not done; DV, diagnostic verification; POS, positive; NEG, negative Green boxes represent concordant results by at least two protocols. For this comparison, the final LymeSpot results that needed diagnostic verification were classified as 'positive', and were subsequently combined with the positive results, as was done for the assessment of the diagnostic performance of the LymeSpot assay in the current manuscript by Van Gorkom et al. [1].

a. The final result of the LymeSpot assay, as described in the instruction manual of the LymeSpot assay, was based on a combination of the stimulation indices of both antigens and was only calculated when the assay was valid (Fig. S2 and the 'Materials and Methods' of the current manuscript of Van Gorkom et al., respectively [1]).
b. For this study participant, a T-cell Xtend step was performed prior to the PBMC isolation as the venipuncture was performed the previous day.
c. The Osp-mix using the old LymeSpot protocol was not tested due to insufficient numbers of PBMCs and this result was therefore missing. Interpretation of the mean SFC count after stimulation with *B. burgdorferi* B31 whole-cell lysate resulted in a positive outcome, and thus the final result is either diagnostic verification (if the mean SFC count in the Osp-mix stimulated wells would result in a negative outcome), or positive (if the mean SFC count in the Osp-mix stimulated wells would result in a positive outcome).
d. The Osp-mix was tested in the singular due to insufficient amounts of PBMCs.

THE FINAL LYMESPOT RESULTS OBTAINED BY USING THE THREE PBMC ISOLATION PROTOCOLS

The majority (varying from 71.4% to 85.3% for the three protocols) of the study participants had a LymeSpot result that either needed diagnostic verification or was positive (Table S4.3). None of the study participants had a negative LymeSpot result for all three protocols.

Comparison of the final LymeSpot results among untreated healthy individuals did not show a difference between the three protocols in case *B. burgdorferi* B31 whole-cell lysate was used to stimulate the PBMCs (raw *P* value = 0.223) (Table S4.3). Similarly, no differences were found between final LymeSpot results for the three protocols among the treated healthy individuals (raw *P* value = 0.368) (Table S4.3).

The final results of the old LymeSpot assay which was active during the study period described in the current manuscript of Van Gorkom et al. [1], and of the in-house *Borrelia* ELISpot protocol were concordant for five cases; two untreated healthy individuals (participants 2 and 3), and three treated healthy individuals (participants 4, 6, and 7) (Table S4.3). For two cases, one untreated healthy individual (participant 1) and one treated healthy individual (participant 5), the old LymeSpot protocol and the in-house *Borrelia* ELISpot protocol yielded conflicting results (Table S4.3).

Comparison of the final results obtained with the new LymeSpot protocol, which is currently active, and the in-house *Borrelia* ELISpot protocol showed that both protocols were concordant for six cases (Table S4.3). For one untreated healthy individual (participant 2), the results obtained by using the new LymeSpot protocol and the in-house *Borrelia* ELISpot protocol were discordant (Table S4.3).

Interestingly, most discordant results were seen between the final results obtained with the old and the new LymeSpot protocol. For four cases, one untreated healthy individual (participant 3), and three treated healthy individuals (participants 4, 6, and 7), the results were concordant (Table S4.3). For three cases, two untreated healthy individuals (participants 1 and 2), and one treated healthy individual (participant 5), the final LymeSpot results by using the old and new LymeSpot protocol were discordant (Table S4.3).

To summarize, the final LymeSpot results obtained with the in-house *Borrelia* ELISPOT protocol always matched the results obtained with at least one of the two protocols recommended by the manufacturer, and most variation was found between the old and new LymeSpot protocol.

DISCUSSION DATA S4

Variations in any protocol can influence the results of the assay concerned. In this pilot study, we investigated the influence of various PBMC isolation protocols on the performance of the LymeSpot assay, and found that the largest number of discordant LymeSpot results was found between the old and the new LymeSpot protocol. However, no significant differences were found between the SFC counts, nor between the final LymeSpot results, for any of the three evaluated PBMC isolation protocols.

One of the differences between our in-house *Borrelia* ELISpot protocol and the two LymeSpot protocols was the method to separate the PBMCs. The Leucosep tubes we used in the in-house *Borrelia* ELISpot protocol also contain Ficoll-paque PLUS (identical to the separation medium used in the old and new LymeSpot protocols using standard tubes), and in addition, a porous barrier

consisting of polyethylene which prevents the mixing of blood with Ficoll. The results of our experiment showed some differences between our in-house *Borrelia* ELISpot protocol and the two LymeSpot protocols; however, no protocol was superior and most differences were found between the results obtained with the old and the new LymeSpot protocol. Other studies also showed that PBMCs isolated by Leucosep tubes performed equally well using an ELISpot assay compared to PBMCs isolated by using standard tubes using a Ficoll-gradient [4, 5].

Variations in centrifugation time and speed as well as variations in the various washing steps of the PBMCs could also have an effect on the performance of the LymeSpot assay. Interestingly, the manufacturer of the LymeSpot assay implemented a number of changes to their recommended PBMC isolation protocol in 2018, most importantly the speed of the various centrifugation steps. In a personal communication, the manufacturer informed us that the PBMC isolation protocol was adjusted upon request by several laboratories who mentioned low PBMC yields by using the old LymeSpot protocol. Comparison of the PBMC yields in our experiment confirmed that the PBMC yield indeed was lower for the old LymeSpot protocol (mean of 2.9 x 10^6 ± 1.4 PBMCs/ml) than for the new LymeSpot protocol (mean of 4.8 x 10^6 ± 1.7 PBMCs/ml). The PBMC yield using our in-house *Borrelia* ELISpot protocol was comparable with the PBMC yield of the new LymeSpot protocol (mean of 5.0 x 10^6 ± 1.1 PBMCs/ml). Although the PBMC yields were different between the old and new LymeSpot protocol, the amount of PBMCs tested per well in the LymeSpot assays was standardized, and, thus, equal for both protocols. Nevertheless, most differences in the final results of the LymeSpot assay were found between the old and new LymeSpot protocol. Since differences consisted of a negative old LymeSpot result versus a positive new LymeSpot result, and vice versa, we do not believe these differences are caused by the differences in speed of the various centrifugation steps and raises questions with regard to the robustness of the assay. A number of studies [4, 6-10] used centrifugation speeds ranging from 300 to 640 *g* for 7 to 10 minutes for the first wash step, and from 300 to 470 *g* for 7 to 10 minutes for the second wash step underlining that a broad range of centrifugation speeds can be used to wash the PBMCs.

Another factor that could have influenced the diagnostic performance of the LymeSpot assay in the current manuscript of van Gorkom et al. [1] was the amount of PBMCs tested per well. For the in-house *Borrelia* ELISpot protocol we tested 2.5 x 10^5 PBMCs/well. In contrast, for the old and new LymeSpot protocols we tested the recommended 2.0 x 10^5 PBMCs/well, each. The amount of PBMCs and the PBMC isolation procedure as described in our in-house *Borrelia* ELISpot protocol (Table S4.3) is similar to the one used in our laboratory for the T-SPOT.TB test (T-SPOT.TB, Oxford Immunotec Ltd., Abingdon, UK). Our laboratory has extensive experience using the T-SPOT.TB test [6, 8, 11], and annual (external) quality controls for the T-SPOT.TB test are always met (data not shown). As expected, we found higher mean SFC counts when using our in-house *Borrelia* ELISpot protocol, although this was not significant and did not result in a higher percentage of positive LymeSpot results. Interestingly, a higher mean SFC count coincided with an increased variation between the mean SFC counts, and was largest for the in-house *Borrelia* ELISpot protocol. The observed association between a higher variation for higher SFC counts has also been described by Smith et al. [12] who evaluated different ELISpot protocols used for the diagnosis of tuberculosis and concluded that the consequence of this variation is limited when cut-offs are established at low SFC counts.

Another critical factor known to influence the ELISpot performance is the time between the venipuncture and the processing of the blood samples, as the numbers of SFCs can decrease over time [12]. For two study participants in this experiment, blood was drawn one day prior to PBMC isolation. For these blood samples, we used a pre-treatment step with T-cell Xtend. Comparison of the final SFC counts for the three protocols showed that increased numbers of SFCs were found for both study participants for at least one *Borrelia* antigen in at least one of the protocols.

Therefore, we conclude that other factors are more critical and that the use T-Cell Xtend is not detrimental for the performance of the LymeSpot assay, which has also been shown by others using much larger study populations [6, 13, 14].

To conclude, although the current experiment is limited by the number of study participants, the results of this pilot study show that the four deviations from the LymeSpot protocol (the PBMC separation medium used, the various centrifugation steps, the medium used to wash the PBMCs, and the amount of PBMCs) are not the most critical steps in the assessment of the diagnostic performance of the LymeSpot assay. When using three different work-up protocols of the LymeSpot assay to test the blood of seven patients, no statistically significant differences were found in the resulting SFC counts and the final LymeSpot results. Based on these observations, we are confident that the conclusion of our study that the LymeSpot assay cannot be used to diagnose Lyme neuroborreliosis is not influenced by the different PBMC isolation protocols we used.

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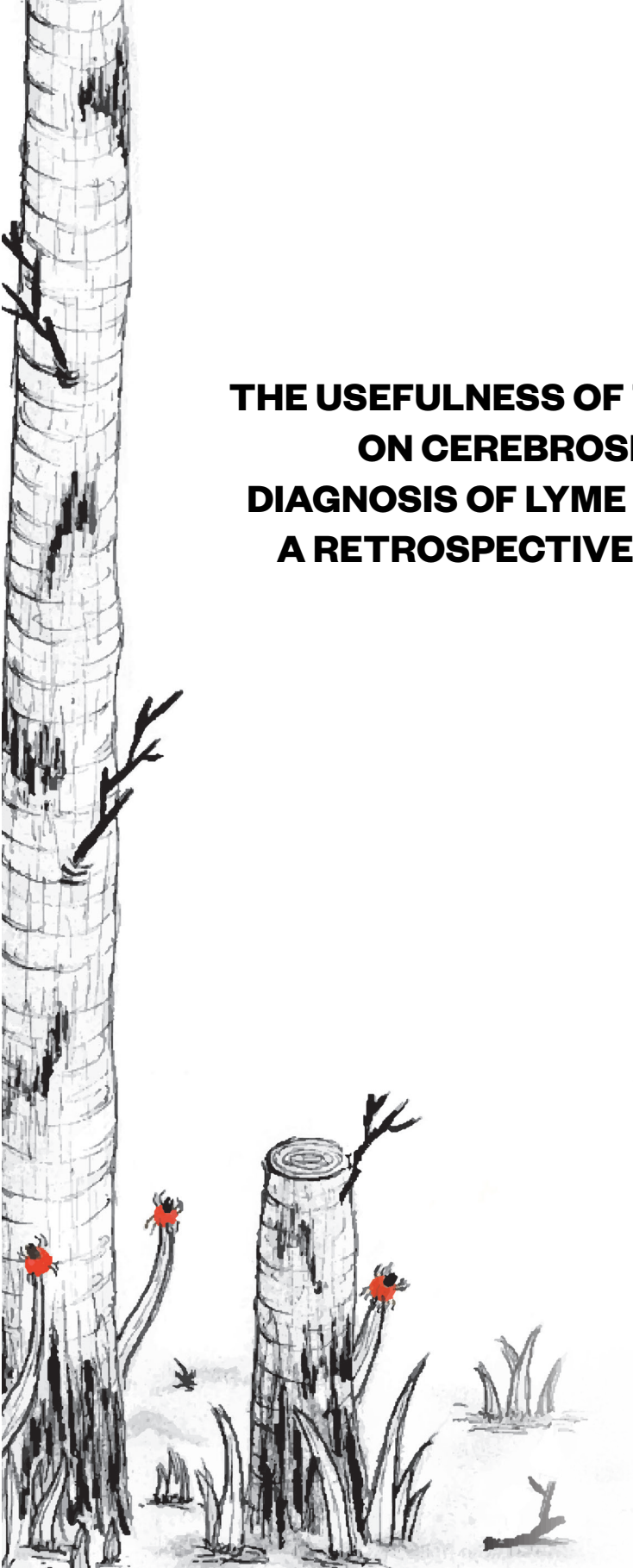

4

THE USEFULNESS OF TWO CXCL13 ASSAYS ON CEREBROSPINAL FLUID FOR THE DIAGNOSIS OF LYME NEUROBORRELIOSIS: A RETROSPECTIVE STUDY IN A ROUTINE CLINICAL SETTING

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ABSTRACT

Recent studies have shown elevated levels of the B-cell chemokine (C-X-C motif) ligand 13 (CXCL13) in the cerebrospinal fluid (CSF) of patients with early Lyme neuroborreliosis (LNB). In this retrospective study, we evaluated the diagnostic performance of the Quantikine CXCL13 enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc., MN, USA) and the *recom*Bead CXCL13 assay (Mikrogen, Neuried, Germany) for the detection of CXCL13 in CSF. All consecutive patients from whom a CSF and a serum sample had been collected between August 2013 and June 2016 were eligible for inclusion. Patients suspected of LNB were classified as definite, possible, or non-LNB according to the guidelines of the European Federation of Neurological Societies (EFNS). Due to the limited number of LNB patients in the predefined study period, additional LNB patients were included from outside this period. In total, 156 patients (150 consecutive patients and 6 additional LNB patients) were included. Seven (4.5%) were classified as definite, eight (5.1%) as possible, and 141 (90.4%) as non-LNB patients. Receiver operating characteristic (ROC) curve analysis comparing definite-LNB patients with non-LNB patients showed a cutoff value of 85.9 pg/ml for the Quantikine CXCL13 ELISA and 252.2 pg/ml for the *recom*Bead CXCL13 assay. The corresponding sensitivity was 100% (95% confidence interval [CI], 100% to 100%) for both, and the corresponding specificities were 98.6% (95% CI, 96.5% to 100%) for the CXCL13 ELISA and 97.2% (95% CI, 93.6% to 100%) for the *recom*Bead CXCL13 assay. This study showed that CXCL13 in CSF can be of additional value for the diagnosis of LNB.

KEYWORDS

Borrelia, Lyme neuroborreliosis, cerebrospinal fluid, CXCL13, Reibergram, blood-CSF barrier functionality, intrathecal antibody synthesis

Lyme borreliosis (LB) is a tick-borne disease caused by spirochetes of the *Borrelia burgdorferi* sensu lato group. The most frequent manifestation of LB is a local skin rash referred to as erythema migrans [1, 2]. When unnoticed, the *Borrelia* bacterium can spread throughout the body and infect organs such as nerves, joints, or heart. Nervous system involvement, also known as Lyme neuroborreliosis (LNB), occurs in about 2 to 13% of LB cases [3-6]. In the Netherlands, the incidence rate of LNB was 2.6 (95% confidence interval [CI], 2.4 to 2.8) per 100,000 inhabitants in 2010, as calculated from a nationwide survey conducted among physicians [4]. Patients with clinical symptoms suggestive of LNB often present with manifestations involving the peripheral and/or cranial nerves, such as radiculopathy and/or cranial neuropathy. The diagnosis of LNB should be supported by laboratory analysis. Detection of the *Borrelia* bacterium by PCR or culture is not very sensitive [7-9]. Instead, the guidelines set by the Federation of Neurological Societies (EFNS) recommend the detection of intrathecally produced *Borrelia*-specific antibodies together with the presence of pleocytosis (≥5 leucocytes/µl) in the cerebrospinal fluid (CSF) [10]. The interpretation of laboratory tests used for the detection of intrathecally produced *Borrelia*-specific antibodies; however, can be complicated for various reasons. First, the levels of intrathecally produced *Borrelia*-specific antibodies may be too low for detection in the early stages of the disease (<6 weeks) [11, 12]. Second, intrathecally produced *Borrelia*-specific antibodies may persist after adequate antibiotic treatment [11, 13]. In case of nonspecific clinical symptoms and pleocytosis, the decision to treat often must be taken before the laboratory results on the intrathecal synthesis of *Borrelia*-specific antibodies become available. This underlines the need for new diagnostic tools for LNB, based on markers with high sensitivity and specificity, especially because early antibiotic treatment has proven to be effective [14, 15].

In 2005, elevated levels of the B-cell chemokine (C-X-C motif) ligand 13 (CXCL13) in CSF samples of LNB patients were detected by using a protein expression array [16] and an enzyme-linked immunosorbent assay (ELISA) [17]. CXCL13 was shown to be involved in the migration of B-lymphocytes to the CSF [18, 19], and in some cases, elevated levels of CXCL13 preceded the synthesis of *Borrelia*-specific antibodies in the CSF [20, 21]. Elevated CSF CXCL13 levels have also been found in the absence of pleocytosis [22, 23]. Since 2005, various studies have confirmed that the detection of CXCL13 in CSF is a useful marker for the diagnosis of early LNB, irrespective of the causative *Borrelia* species [24], even though elevated CSF CXCL13 levels have also been found in patients with bacterial [14, 24-29] and viral [21, 25-28, 30-33] central nervous system (CNS) infections, autoimmune diseases [19, 28, 34], and white blood cell line malignancies [26, 35]. As CSF CXCL13 levels decline rapidly after successful antibiotic therapy for LNB, this marker can be used to distinguish an active LNB infection from a previous, cleared infection [16, 20, 25, 28].

Several commercial assays for the detection of CXCL13 are available, and various studies have been performed to investigate the usefulness of CXCL13 in CSF as a marker for the diagnosis of LNB. One of the assays studied the most is the human B-lymphocyte chemoattractant/B cell-attracting chemokine 1 (BLC/BCA-1) immunoassay (Quantikine CXCL13 ELISA; R&D Systems, Inc., Minneapolis, MN, USA). The instruction manual for this assay, however, does not mention the use of CSF for the detection of CXCL13 and consequently lacks a cutoff value for CXCL13 in the CSF. Various studies have shown the potential of the detection of CXCL13 in the CSF for the diagnosis of LNB; however, a broad range of cutoff values was found, ranging from 18 pg/ml to 1,229 pg/ml [14, 20, 21, 23, 25, 26, 28, 36, 37]. The diagnostic performance of a test largely depends on the study design (e.g., prospective versus retrospective and/or case-control versus cross-sectional) [38, 39]. For most studies, the study population did not match a routine clinical setting, and this is a drawback of many LB test evaluation studies [38]. Therefore, the aim of our study was to assess the diagnostic potential of two CXCL13 assays on CSF from all consecutive patients that visited our hospital in a defined time frame and from whom a blood sample was

taken as well. We chose an established CXCL13 assay (i.e., the Quantikine CXCL13 ELISA [R&D systems, Inc.]) to evaluate the performance of the *recom*Bead CXCL13 assay (Mikrogen GmbH, Neuried, Germany), which makes use of the innovative Luminex xMAP technology.

MATERIALS AND METHODS

STUDY POPULATION

Retrospectively, and irrespective of their clinical suspicion, all consecutive patients from whom a CSF and a blood sample (drawn within 24 h of the lumbar puncture [LP]) had been sent to the microbiology laboratory of the Diakonessenhuis Hospital, Utrecht, the Netherlands, between August 2013 and June 2016 were selected. The patients were divided into three groups based on the criteria for LNB as defined by the EFNS (10). These criteria are (i) the presence of neurological symptoms suggestive of LNB in the absence of other possible causes, (ii) CSF pleocytosis (≥5 leukocytes/μl), and (iii) intrathecal synthesis of *Borrelia*-specific antibodies. If all criteria were met, then the patient was classified as a definite-LNB patient; if two criteria were met, then the patient was classified as a possible-LNB patient. In all other cases, the patient was classified as a non-LNB patient. As the samples that were collected within the framework of this study were also used for another study that evaluated various commercial LNB assays (T. van Gorkom, W. Voet, G. H. J. van Arkel, M. Heron, S. F. T. Thijsen, and K. Kremer, unpublished data), the CSF and serum sample pairs were only included if at least 1,250 ml of CSF and 110 ml of serum were available (Fig. 1). Patients with hemolytic CSF or who were treated intravenously with IgG were excluded from the study, as both could lead to erroneous results [40, 41]. For all study participants, information about symptoms, symptom duration prior to the LP, and antibiotic treatment for LNB was assessed through consultation of the electronic patient files. No systematic survey was conducted on the use of antibiotic treatment in the period preceding the LP.

Due to the relatively low number of LNB patients with sufficient material in the predefined study period, additional LNB patients (definite or possible LNB) were chosen from outside the study period (from February 2011 to July 2013 and from July 2016 to November 2017). The LNB patients who were eligible had taken part in two other studies of our research group [42, 43], and consequently, both CSF and serum from the time of diagnosis had been stored. For these additional LNB patients, the same inclusion criteria applied as for all consecutive patients. All samples used in this study were anonymized. According to the rules of our hospital, our study was exempt from approval of the local Ethics Committee because the main goal was to validate a new assay for the diagnosis of LNB for which leftover material could be used. We did, however, obtain approval from the hospital board to conduct this study.

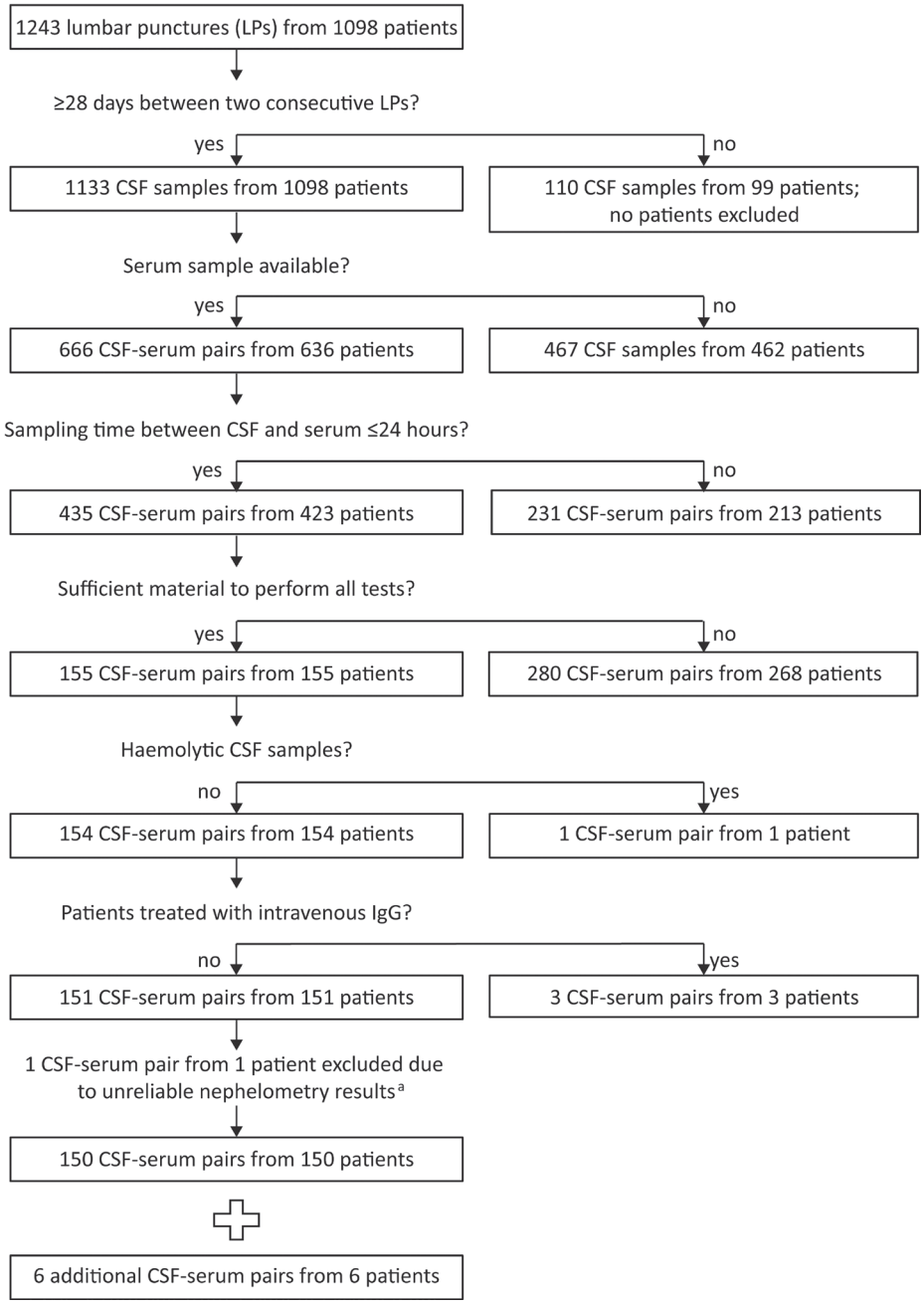

Fig. 1. Flow chart of the inclusion of the 156 patients. From all 1,098 consecutive patients with a lumbar puncture in the period between August 2013 and June 2016, 155 (14.1%) had sufficient amounts of CSF and serum that were drawn from the patient within 24 h. Five (3.2%) of the 155 patients were excluded for various reasons. CSF-serum pairs from six additional LNB patients were collected between February 2011 and July 2013 (n = 1) or between July 2016 and November 2017 (*n* = 5). [a]Initially, testing had started with 151 CSF-serum pairs; however, one CSF-serum pair was excluded after the nephelometry results became available. The albumin concentration in the CSF of this CSF-serum pair far exceeded the total protein concentration in CSF, which was measured at the time of diagnosis, 3 months earlier, implying a sample mix-up.

DETECTION OF *BORRELIA*-SPECIFIC ANTIBODIES

The detection of *Borrelia*-specific total immunoglobulin (IgM and IgG) in serum was done by using the C6 enzyme-linked immunosorbent assay (ELISA) (Immunetics, Boston, MA, USA), and equivocal and positive C6 ELISA results were confirmed by using the *recom*Line IgM and IgG immunoblot test (Mikrogen Diagnostik GmbH) as described previously [43]. The detection of intrathecally produced *Borrelia*-specific IgM and IgG was done by using the second-generation IDEIA LNB assay (Oxoid, Basingstoke, United Kingdom) as described previously [43]. To determine the intrathecal synthesis of *Borrelia*-specific IgM (or IgG), CSF and serum of a CSF-serum pair were tested simultaneously in the same run. The IDEIA LNB assay is based on the capture ELISA principle and determines the fraction of *Borrelia*-specific IgM (or IgG) as part of the total amount of IgM (or IgG) in CSF and serum. If the fraction of *Borrelia*-specific IgM (or IgG) in the CSF exceeds the fraction in serum, then intrathecal synthesis of *Borrelia*-specific IgM (or IgG) is proven. Consequently, a correction for a dysfunctional blood-CSF barrier was not needed.

BORRELIA SPECIES PCR ON CSF

An in-house real-time *Borrelia* species PCR was used, for which the 23S rRNA gene was used as a target. A total of 190 ml of the CSF sample was processed together with an internal control consisting of *Synechococcus* DNA (Dutch Institute of Ecology/Royal National Academy of Sciences, Yrseke, The Netherlands) by using the MagnaPure LC total-nucleic acid, high-performance isolation kit (Roche Diagnostics, Almere, The Netherlands) on a MagnaPure LC instrument (Roche Diagnostics). The amount of internal control used had to result in a threshold cycle value in the diagnostic range, and 10 µl was sufficient, which was established empirically. The isolated DNA was eluted in 100 µl of elution buffer (Roche Diagnostics). The real-time PCR contained 12.5 µl TaqMan universal PCR mastermix (ThermoFisher Scientific, Bleiswijk, the Netherlands), 10 µl of eluted DNA, and 2.5 µl of a primer and probe mix. The primer and probe mix consisted of a 300 nM concentration of the forward primer (5'-GGGCGATTTAGTTAGATGTGGTAGA-3'), a 900 nM concentration of the reverse primer (5'-CAAGCTTCAGCCTGGCCATA-3'), and a 200 nM concentration of the probe (5'-6-carboxyfluorescein [FAM]-AASCCGAGTGATCTAT-3' minor groove binder [MGB] nonfluorescent quencher). The *Borrelia* species PCR was performed in a 96-well plate (ThermoFisher Scientific) on an ABI 7500 system (ThermoFisher Scientific). The program consisted of 1 cycle of 2 min at 50°C, 1 cycle of 15 min at 95°C, and 45 cycles that consisted of 15 s at 95°C followed by 1 min at 60°C. A positive control (DNA of *B. burgdorferi*; Vircell Microbiologists, Granada, Spain) and a negative control (elution buffer; Roche Diagnostics) were included in each experiment to control for amplification and contamination.

DETECTION OF CXCL13 IN CSF

CSF samples of all patients were tested by using two CXCL13 assays according to the manufacturer's instructions. The first assay was the Quantikine CXCL13 ELISA (R&D Systems, Inc.), hereinafter referred to as the CXCL13 ELISA, which was performed on a Dynex DS2 automated ELISA instrument (Dynex Technologies, Chantilly, VA, USA) and analyzed with the DS-Matrix software (Dynex Technologies). The manufacturer of this CXCL13 ELISA recommends the assay for use in the quantitative measurement of CXCL13 in cell culture supernatants, serum, plasma, and saliva, but does not mention its use for CSF [44]. The final CSF CXCL13 concentrations (in pg/ml) were calculated by using a standard curve. The standard curve was based on seven standards with concentrations ranging from 7.81 pg/ml to 500 pg/ml. These standards were included in each run, and after the optical densities (ODs) were measured, a curve was constructed by plotting the mean OD value from each standard on the y-axis and the concentration on the x-axis as a log/log graph in the Dynex DS2 automated ELISA instrument (Dynex Technologies). Subsequently, the OD values from the CSF samples were interpreted by using this standard curve. Concentrations below the lower detection limit of the standard curve (<7.81 pg/ml) were assigned a value of 7.81 pg/ml, and concentrations above the upper detection limit of the standard curve (>500 pg/

ml) were assigned a value of 500 pg/ml.

The second assay was the Luminex-based *recom*Bead CXCL13 assay (Mikrogen Diagnostik GmbH). CXCL13 concentrations (in pg/ml) were measured by using the Luminex xMAP technology. Bead analysis was done on a Bio-Plex 200 instrument (Bio-Rad Laboratories, Hercules, CA, USA) by using the Bio-Plex Manager software, version 6.1 (Bio-Rad Laboratories). The final CXCL13 concentration of a CSF sample was calculated by using a calibrator, together with the batch-dependent 4-parameter logistic (4-PL) coordinates of a standard curve by using the *recom*Quant evaluation software version 4.9.5 (Mikrogen Diagnostik GmbH). The measurement range is located between 9.00 and 1,000 pg/ml. CXCL13 concentrations of less than 9.00 pg/ml were assigned a value of 9.00 pg/ml, and CXCL13 concentrations of more than 1,000 pg/ml were assigned a value of 1,000 pg/ml. According to the instruction manual, CSF CXCL13 concentrations of less than 190 pg/ml are normal, between 190 and 300 pg/ml are borderline, and more than 300 pg/ml are elevated, for which an active LNB infection is suspected if symptoms match [41].

BLOOD-CSF BARRIER FUNCTIONALITY, INTRATHECAL TOTAL-ANTIBODY SYNTHESIS, AND CONSTRUCTION OF REIBERGRAMS

Blood-CSF barrier functionality and intrathecal total-antibody synthesis were assessed by the construction of Reibergrams [45]. Therefore, CSF and serum concentrations of albumin, total IgM, and total IgG were determined by nephelometry using the BN ProSpec system (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) following the instructions of the manufacturer. By using the website www.albaum.it [46], these concentrations were used to calculate the CSF/serum quotients for albumin (Q Alb), IgM (Q IgM), and IgG (Q IgG), as well as the intrathecal fractions of IgM and IgG. Subsequently, the Q IgM and Q IgG of all patients were plotted relative to the Q Alb. In both the IgM and the IgG Reibergram, the reference ranges for the blood-derived fractions of total IgM and total IgG were shown by nonlinear hyperbolic curves (mean reference line ± 3 standard deviations). These lines best reflect the biological laws of diffusion from the blood as has been described by Reiber [47]. In addition, four lines that are located above the upper reference line show 20%, 40%, 60%, and 80% increases of the measured total-antibody concentration in the CSF and thus reflect the fraction of intrathecally produced total antibodies [45]. A vertical line can be added to show the age-dependent reference line for Q Alb. In this study, only the mean age-dependent reference line for Q Alb is reported.

Based on the position of Q IgM and Q IgG in the Reibergram, every patient was classified in one of five groups corresponding to the five areas of the Reibergram [45], as follows: area 1, normal CSF findings (total-antibody quotient below the upper reference line for blood-derived total antibodies and left of the age-dependent reference line for Q Alb); area 2, dysfunctional blood-CSF barrier (total-antibody quotient below the upper reference line for blood-derived total antibodies and right of the age-dependent reference line for Q Alb); area 3, dysfunctional blood-CSF barrier and intrathecal total-antibody synthesis (total-antibody quotient above the upper reference line for blood-derived total antibodies and right of the age-dependent reference line for Q Alb); area 4, normal blood-CSF barrier and intrathecal total-antibody synthesis (total-antibody quotient above the upper reference line for blood-derived total antibodies and left of the age-dependent reference line for Q Alb); and area 5, indicative of a methodological error (e.g., unpaired CSF/serum samples, measurement in antigen excess range, etc.). The overall Reibergram classification was based on the combined IgM and IgG results. There was no proof of intrathecal total-antibody synthesis if both the intrathecal total-IgM and the intrathecal total-IgG fraction were equal to or less than 10%. If either one or both intrathecal fractions were larger than 10%, then intrathecal total-antibody synthesis was proven [45].

DATA HANDLING AND STATISTICAL ANALYSIS

For all statistical analyses, Rstudio (version 1.3.959, 2009-2020) was used [48]. The data were analyzed by performing two-group comparisons, for which the coin package was used [49]. Nominal data were analyzed by using either the exact Pearson's chi-squared test or the approximate Monte Carlo resampling (10^6) Pearson's chi-squared test, and quantitative data were analyzed by using the exact Wilcoxon-Mann-Whitney test. Results are shown as the (geometric) mean value with the 95% CI or as the median value with the range. For construction of the dot plots, GraphPad Prism (version 8.4.1; GraphPad Software, San Diego, CA, USA) was used.

To assess the diagnostic performance of both CXCL13 assays for the diagnosis of LNB, a receiver operating characteristic (ROC) curve was constructed for each assay separately and used to calculate the area under the curve (AUC) by using the pROC package [50]. Therefore, the CXCL13 concentrations in CSF samples of cases (i.e., definite-LNB patients) were compared with those of controls (i.e., non-LNB patients). Subsequently, the optimal cutoff values were calculated by using the point on the ROC curve for which the distance to the upper left corner, where both sensitivity and specificity are 100%, was shortest, and was determined by the square root of $[(1\text{-sensitivity})^2 + (1\text{-specificity})^2]$. CSF CXCL13 levels above the optimal cutoff value in each assay were classified as positive. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with 95% CIs for each assay were calculated by using the optimal cutoff value. The performances of both assays were compared by comparison of the ROC curves using the clinfun package and 10^6 permutations [51].

Raw *P* values of <0.050 were statistically significant; however, they were interpreted after correction for the multiple statistical analyses in this study, for which the Benjamini-Hochberg procedure (BH) was applied [52]. The false discovery rate (FDR) was set at the level of 5.0%, and less than one false-positive result was allowed in our list of rejections.

RESULTS

STUDY POPULATION

A flow chart of the study population is shown in Fig. 1. The final study population comprised 156 patients, of whom 150 (96.2%) consisted of all consecutively collected patients who had had an LP in our hospital between August 2013 and June 2016 and from whom sufficient amounts of CSF and serum were collected. These 150 consecutive patients did not significantly differ regarding age and gender from the 1,098 consecutive patients for whom at least one LP was performed in the predefined study period (data not shown). Six additional LNB patients, who were part of two other studies from our research group [42, 43], were selected from outside this period, to increase the number of LNB patients. In total, 78 men and 78 women were included in the study. The mean age of the patients was 52.5 years (95% CI, 49.9 to 55.1), the geometric mean CSF cell count was 1.6 cells/µl (95% CI, 1.1 to 2.3 cells/µl), and the geometric mean symptom duration was 60.3 days (95% CI, 43.0 to 84.0 days). The basic characteristics for each study group are shown in Table 1. As expected, considering the definitions of definite-LNB and possible-LNB cases (10), LNB patients, more often than non-LNB patients, had pleocytosis and *Borrelia*-specific antibodies in their blood. Definite-LNB patients had intrathecally produced *Borrelia*-specific antibodies more often than possible-LNB patients and non-LNB patients (Table 1).

DEFINITE-LNB PATIENTS

Seven (4.5%) of the 156 patients fulfilled the three criteria for definite LNB set by the EFNS (10) (Table 1). Two (28.6%) of them had a positive *Borrelia* species PCR result (Table 1). Six (85.7%) of the seven definite-LNB patients had radiculopathy, of whom two also had cognitive impairment.

One patient also had cranial and peripheral neuropathy. The remaining patient had myelitis transversa. All definite-LNB patients were treated with ceftriaxone (2 g/day) intravenously for either 14 (*n* = 4) or 28 (*n* = 3) days, following the Dutch guidelines for LB (52); the treatment was started after the LP was performed (data not shown).

Table 1. Demographic and clinical characteristics among all 156 patients included in this study

	Value for indicated patient group[b]				Raw *P* value for BH comparison[e]		
Characteristic[a]	**All patients (*n* = 156)**	**dLNB (*n* = 7)[c]**	**pLNB (*n* = 8)[d]**	**Non-LNB (*n* = 141)**	**(1 vs 2)[c]**	**(1 vs 3)[d]**	**(2 vs 3)[e]**
Gender [no. of males (%)]	78 (50.0)	6 (85.7)	4 (50.0)	68 (48.2)	0.282	0.116	1.000
Age [mean (95% CI)]	52.5 (49.9-55.1)	68.8 (61.0-76.2)	52.1 (37.9-66.4)	51.8 (49.0-54.5)	0.094	0.007[f]	0.776
Pleocytosis							
≥5 leucocytes/µl [no. (%)]	36 (23.1)	7 (100)	7 (87.5)	22 (15.6)	1.000	< 0.001[f]	< 0.001[f]
CSF cell count/µl [geometric mean (95% CI)]	1.6 (1.1-2.3)	67.4 (27.4-164)	24.5 (8.9-66.7)	1.1 (0.8-1.6)	0.179	< 0.001[f]	< 0.001[f]
Positive *Borrelia* species PCR on CSF	2 (1.3)	2 (28.6)	0 (0.0)	0 (0.0)	0.200	0.002[f]	1.000
Intrathecal *Borrelia*-specific Ab synthesis [no. (%)]	8 (5.1)	7 (100)	1 (12.5)	0 (0.0)	0.001[f]	< 0.001[f]	0.054
Detection of *Borrelia*-specific Abs in blood [no. (%)]	44 (28.2)	7 (100)	6 (75.0)	31 (22.0)	0.467	< 0.001[f]	0.003[f]
Duration of symptoms in days [geometric mean (95% CI)]	60.3 (43.0-83.9)	26.1 (11.0-62.2)	31.8 (11.8-85.6)	64.7 (45.2-93.7)	0.955	0.118	0.182

a. CI, confidence interval; CSF, cerebrospinal fluid; Ab, antibody.
b. Patients are categorized as definite-Lyme neuroborreliosis (dLNB), possible-LNB (pLNB), or non-LNB cases based on the EFNS criteria [10].
c. Three of 7 (42.9%) dLNB patients were selected between August 2013 and June 2016, and 4/7 (57.1%) were from outside this period.
d. Six of 8 (75.0%) pLNB patients were selected between August 2013 and June 2016, and 2/8 (25.0%) were from outside this period.
e. BH, Benjamini-Hochberg: 1 versus 2, definite- versus possible-LNB patients; 1 versus 3, definite- versus non-LNB patients; and 2 versus 3, possible versus non-LNB patients.
f. Significant *P* value after applying the Benjamini-Hochberg procedure (FDR ≤ 5.0%).

POSSIBLE-LNB PATIENTS

Eight (5.1%) of the 156 patients fulfilled two of the three EFNS criteria for definite LNB (10) and, thus, were classified as possible-LNB patients (Table 1). None of these patients had a positive *Borrelia* species PCR result (Table 1). LNB-specific symptoms among the possible-LNB patients included radiculopathy (*n* = 1; 12.5%), cranial neuropathy (*n* = 6; 75.0%), or peripheral neuropathy (*n* = 1; 12.5%). All of the possible-LNB patients were treated for LNB according to the Dutch guidelines for LB [53]; the treatment was started after the LP was performed (data not shown). Six (75.0%) of the possible-LNB patients received ceftriaxone (2 g/day) intravenously for either 14 (*n* = 5) or 28 (*n* = 1) days. One possible-LNB patient had started with intravenous ceftriaxone (2 g/day) but after 5 days switched to oral doxycycline (100 mg twice a day) for 25 days because of an allergic reaction. The remaining possible-LNB patient received oral doxycycline (100 mg twice a day) from the start for 30 days (data not shown).

NON-LNB PATIENTS

One hundred forty-one (90.4%) of the 156 patients were classified as non-LNB patients, as they did not fulfil at least two of the three EFNS criteria for definite LNB (Table 1) [10]. None of them had a positive *Borrelia* species PCR result (Table 1). The five non-LNB patients for whom the CSF cell count was the highest (range, 39 to 821 cells/µl) were diagnosed with neurosyphilis

(*n* = 1), *Streptococcus* meningitis (*n* = 1), tuberculous meningitis (*n* = 1), and viral meningitis (*n* = 2). Other diagnoses that were found at least twice among the non-LNB patients included peripheral neuropathy (*n* = 22), demyelinating conditions (*n* = 17) that included seven cases of multiple sclerosis, radiculopathy (*n* = 9), non-CSF infectious disease (*n* = 8), spinal stenosis (*n* = 7), (transient) facial nerve paralysis (*n* = 6), nonspecific tendon-myogenic pain (*n* = 4), cerebrovascular accident (*n* = 3), microvascular white matter lesions (*n* = 3), headache/migraine (*n* = 3), cancer (*n* = 3), residual complaints after a previous CSF infection (*n* = 3; LNB, neurosyphilis, and viral meningitis), epilepsy (*n* = 2), sleep disorder (*n* = 2), psychogenic disorder (*n* = 2), and arthralgia (*n* = 2). Eighteen non-LNB patients had a diagnosis that was found to be unique in this study population; for 22 non-LNB patients, a diagnosis was never established (data not shown).

CXCL13 CONCENTRATIONS IN THE CSF

Overall, the median CSF CXCL13 concentrations measured by using the *recom*Bead CXCL13 assay were higher than those measured by using the CXCL13 ELISA. Both assays, however, showed similar distributions between the three EFNS groups [10], with the highest CSF CXCL13 levels among the definite-LNB patients (Fig. 2). Using the CXCL13 ELISA, the CSF CXCL13 concentrations among the definite-LNB patients ranged from 138 to 500 pg/ml, with a median CSF CXCL13 concentration of 500 pg/ml (Fig. 2A). This was higher than the median CSF CXCL13 concentration of 30.9 pg/ml (range, 7.81 to 500 pg/ml) among the possible-LNB patients, but not significant (*P* = 0.044; FDR > 5.0%). The median CSF CXCL13 concentration among the non-LNB patients was 7.81 pg/ml (range, 7.81 to 500 pg/ml), and this was significantly lower (*P* < 0.001; FDR ≤ 5.0%) than among both the definite-LNB and the possible-LNB patients (Fig. 2A).

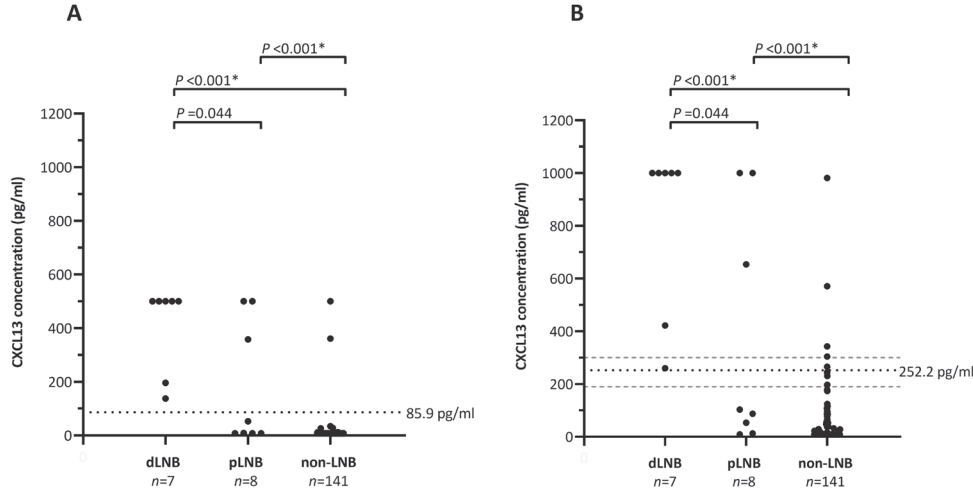

Fig. 2. CSF CXCL13 concentrations among seven definite-Lyme neuroborreliosis (dLNB) patients, eight possible-Lyme neuroborreliosis (pLNB) patients, and 141 non-Lyme neuroborreliosis (non-LNB) patients determined by using the CXCL13 ELISA (A) and the *recom*Bead CXCL13 assay (B). The *P* values displayed are raw *P* values that were found to be either significant (FDR ≤ 5.0%; marked with an asterisk) or not (FDR > 5.0%) after applying the Benjamini-Hochberg procedure to account for the multiple comparisons in this study. The (black) dotted horizontal lines in panels A and B are the cutoff values calculated by using ROC curve analysis. The (gray) dashed horizontal lines in panel B are the cutoff values based on the instruction manual of the manufacturer (negative, <190 pg/ml [below lower dashed line]; borderline, 190 to 300 pg/ml [between the dashed lines]; and positive, >300 pg/ml [above upper dashed line]) [41].

The CSF CXCL13 concentrations among definite-LNB patients measured by using the *recom*Bead CXCL13 assay ranged from 260 to 1,000 pg/ml, with a median of 1,000 pg/ml (Fig. 2B). Among possible-LNB patients, the median CSF CXCL13 concentration was 94.9 pg/ml (range, 9.00 to

1,000 pg/ml), which was lower than the median CSF CXCL13 concentration among definite-LNB patients, although this was not significant (P = 0.044; FDR > 5.0%) (Fig. 2B). The median CSF CXCL13 concentration of 9.00 pg/ml (range, 9.00 to 982 pg/ml) among the non-LNB patients was the lowest and was significantly lower (P < 0.001; FDR ≤ 5.0%) than among both the definite-LNB and possible-LNB patients (Fig. 2B).

DIAGNOSTIC PERFORMANCE OF THE CXCL13 ASSAYS

To assess the diagnostic performance of the CXCL13 ELISA and the *recom*Bead CXCL13 assay, ROC curves were created by comparison of the CXCL13 concentrations among cases (i.e., definite-LNB patients) with those among controls (i.e., non-LNB patients). For both assays, the optimal cutoff value was assessed by using the point on the ROC curve for which the distance to the upper left corner, where both sensitivity and specificity are 100%, was shortest. For the CXCL13 ELISA, the optimal cutoff value was determined to be 85.9 pg/ml, with a sensitivity of 100% and a specificity of 98.6%. For the *recom*Bead CXCL13 assay, the optimal cutoff value was determined to be 252.2 pg/ml, with a sensitivity of 100% and a specificity of 97.2% (Table 2). This optimal cutoff value fell within the borderline range (190 to 300 pg/ml) recommended by the manufacturer of the *recom*Bead CXCL13 assay [41]. The NPV was 100% for both assays, and the PPV was 77.8% for the CXCL13 ELISA and 63.6% for the *recom*Bead CXCL13 assay. No significant difference (P = 0.048; FDR > 5.0%) was found between the diagnostic performance of the two assays (Table 2).

Table 2. Diagnostic performance of the CXCL13 ELISA and the *recom*Bead CXCL13 assay in distinguishing definite-LNB patients from non-LNB patients

Assay	Value (95% CI) for[a]						Raw *P* value for BH comparison
	Threshold (pg/ml)	AUC	% sensitivity	% specificity	PPV (%)	NPV (%)	
CXCL13 ELISA	85.9 (74.7-430.4)	0.993 (0.983-1.000)	100 (100-100)	98.6 (96.5 - 100)	77.8 (58.3 - 100)	100 (100-100)	0.048[b]
*recom*Bead CXCL13 assay	252.2 (228.1-990.8)	0.993 (0.981-1.000)	100 (100-100)	97.2 (93.6 - 100)	63.6 (43.8 - 100)	100 (100-100)	

a. n = 7 definite-LNB patients and n = 141 non-LNB patients; CI, confidence interval; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; BH, Benjamini-Hochberg.
b. Nonsignificant P value after applying the Benjamini-Hochberg procedure (FDR > 5.0%).

INTERPRETATION OF CXCL13 RESULTS

Twelve (7.7%) of the 156 patients included in this study had a positive CSF CXCL13 result in both assays, which included all (n = 7; 100%) definite-LNB patients, three (37.5%) possible-LNB patients, and two (1.4%) non-LNB patients. Five (71.4%) of the seven definite-LNB patients had CSF CXCL13 concentrations above the upper detection limits in both assays. The duration of their symptoms ranged from 21 to 108 days. In contrast, the two definite-LNB patients with shorter durations of symptoms had lower CXCL13 concentrations (both within the detection limits in the two assays). One of them, who had CSF CXCL13 concentrations of 138 pg/ml in the CXCL13 ELISA and 260 pg/ml in the *recom*Bead CXCL13 assay, had had symptoms for 3 days. The other had had symptoms for 14 days and had CSF CXCL13 concentrations of 196 pg/ml in the CXCL13 ELISA and 421 pg/ml in the *recom*Bead assay.

The three possible-LNB patients with positive CSF CXCL13 results in both assays all had pleocytosis. For two of them, the CSF CXCL13 concentrations in both assays exceeded the upper detection limits of the assays. The third possible-LNB patient had CSF CXCL13 concentrations of 358 pg/ml in the CXCL13 ELISA and 654 pg/ml in the *recom*Bead CXCL13 assay. This patient had a shorter duration of symptoms (22 days) than the other two patients (47 and 174 days, respectively).

The two non-LNB patients who had positive CSF CXCL13 results in both assays were the ones

diagnosed with neurosyphilis and tuberculous meningitis. These two patients represented 1.4% of all non-LNB patients. The patient with tuberculous meningitis had CSF CXCL13 concentrations of 361 pg/ml in the CXCL13 ELISA and 571 pg/ml in the *recom*Bead CXCL13 assay. The patient with neurosyphilis had CSF CXCL13 concentrations of 500 pg/ml in the CXCL13 ELISA and 982 pg/ml in the *recom*Bead CXCL13 assay. Three (2.1%) of the 141 non-LNB patients had a positive CSF CXCL13 result in the *recom*Bead CXCL13 assay only (Fig. 2B). These three patients had a normal blood-CSF barrier, no intrathecal total-antibody synthesis (Reibergram area 1) (Fig. 3B), and no pleocytosis. They were diagnosed with (i) a cardiovascular accident and sepsis, (ii) peripheral neuropathy, and (iii) femoral neuropathy.

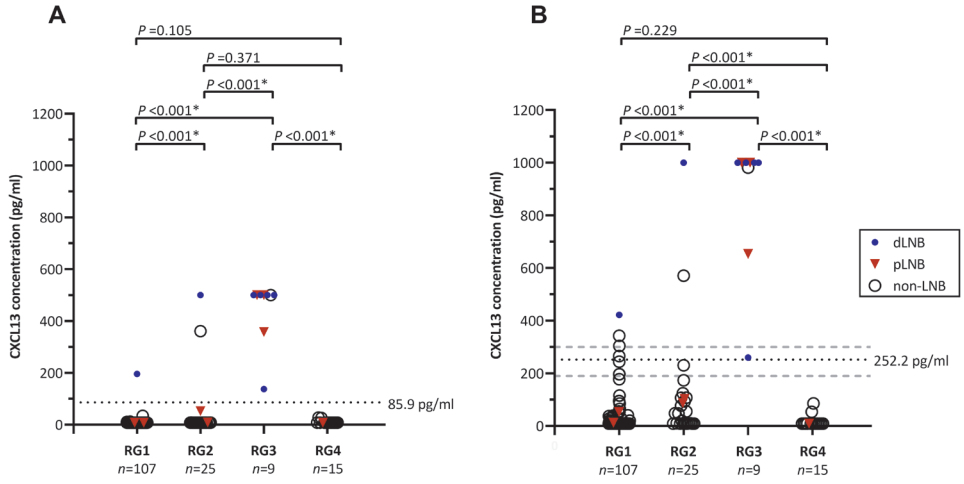

Fig. 3. CSF CXCL13 concentrations in the CSF among the 156 patients included in the study, measured by using either the CXCL13 ELISA (A) or the *recom*Bead CXCL13 assay (B), are grouped by Reibergram classification [45]. The likelihood of patients having Lyme neuroborreliosis (LNB) as defined by the EFNS [10] is marked as shown in the key in panel B: dLNB, definite-LNB patients; pLNB, possible-LNB patients; and non-LNB, non-LNB patients. The Reibergram classification is based on the combined IgM and IgG Reibergrams (Fig. S1) as shown in Table S2 (RG1, Reibergram group 1; RG2, Reibergram group 2; RG3, Reibergram group 3; and RG4, Reibergram group 4). The *P* values displayed are raw *P* values that were either found significant (FDR ≤ 5.0%; marked with an asterisk) or not (FDR > 5.0%) after applying the Benjamini-Hochberg procedure to account for the multiple comparisons in this study. The (black) dotted horizontal lines in panels A and B are the cutoff values calculated by using ROC curve analyses. The (gray) dashed horizontal lines in panel B are the cutoff values based on the instruction manual of the manufacturer (negative, <190 pg/ml [below lower dashed line]; borderline, 190 to 300 pg/ml [between the dashed lines]; and positive, >300 pg/ml [above upper dashed line)] [41].

CSF CXCL13 CONCENTRATION VERSUS BLOOD-CSF BARRIER FUNCTIONALITY AND INTRATHECAL TOTAL-ANTIBODY SYNTHESIS

The construction of the Reibergrams [46] provided insight into the functionality of the blood-CSF barrier and the origin of the total IgM and IgG that was detected in the CSF (Fig. S1 in the supplemental material). Patients were subsequently classified in one of four of the five groups based on their position in the Reibergram (Table S2). Analogous to the EFNS groups described above, the CSF CXCL13 concentrations among the four Reibergram groups showed a similar distribution for both CXCL13 assays (Fig. 3).

A total of 107 (68.6%) patients had a normal blood-CSF barrier in the absence of intrathecal total-antibody synthesis and, thus, were classified in Reibergram group 1 (Fig. 3). Only one (0.9%) of them, a definite-LNB patient, had a positive CSF CXCL13 result in both assays. Twenty-five (16.0%) of the 156 patients had a disturbed blood-CSF barrier in the absence of intrathecal total-antibody synthesis and were consequently classified in Reibergram group 2 (Fig. 3). Two (8.0%)

of them had elevated CSF CXCL13 levels; one was diagnosed with definite LNB, the other one was the one diagnosed with tuberculous meningitis. The highest CSF CXCL13 levels were found among patients in Reibergram group 3 (Fig. 3). These levels were significantly higher ($P < 0.001$; FDR ≤ 5.0%) than the levels in the other three groups. Nine (5.8%) of the 156 patients were classified in this group, as they had a dysfunctional blood-CSF barrier and intrathecally produced total antibodies. Six of these nine patients had intrathecal total-antibody synthesis of both the IgM and the IgG class (Table S2). Five of them were diagnosed with definite LNB, and the other patient was the one diagnosed with neurosyphilis. The remaining three patients were diagnosed with possible LNB, and all of them had intrathecal total-antibody synthesis of the IgM class only. Fifteen (9.6%) of the 156 patients had a normal blood-CSF barrier and proof of intrathecal total-antibody synthesis; however, none of them had elevated CSF CXCL13 levels in either of the CXCL13 assays (Reibergram group 4) (Fig. 3). None of the patients in the study were classified in Reibergram group 5.

DISCUSSION

In this retrospective study, we evaluated the diagnostic potential of the chemokine CXCL13 in diagnosing LNB among all consecutive patients who had had an LP in the routine clinical setting of our hospital and who fulfilled the inclusion criteria. Two assays were used to measure the CXCL13 concentrations in CSF, the CXCL13 ELISA and the *recom*Bead CXCL13 assay. Significantly higher CSF CXCL13 levels were found among definite-LNB patients than among non-LNB patients ($P < 0.001$; FDR ≤ 5.0%). Our study results are in accordance with the results published by other research groups [20, 23, 36, 37] and confirm the usefulness of CXCL13 detection in CSF as a marker in the diagnosis of LNB.

Our study suggests that CXCL13 can be of added value for patients classified as possible-LNB patients based on the EFNS guidelines [10]. The classification of possible-LNB cases can be a matter of dispute, as this is based on clinical symptoms and either pleocytosis or intrathecal *Borrelia*-specific antibody synthesis. Various studies report higher CSF CXCL13 levels for possible-LNB patients with pleocytosis than with intrathecal *Borrelia*-specific antibody synthesis [14, 36], which we also find in our study. It has been suggested that possible-LNB patients with pleocytosis represent early LNB cases for whom the antibody response still had undetectable levels and that possible-LNB patients with intrathecal *Borrelia*-specific antibody synthesis most likely have had a previous LNB or possibly another disease [14, 36]. Long-term persistence of *Borrelia*-specific antibodies in the CSF after successful treatment of LNB has been described [11, 13]. In the current study, eight possible-LNB patients were included, of whom three (37.5%) had a positive CSF CXCL13 result in both assays. These three patients had pleocytosis and, following the aforementioned rationale, most likely had an early LNB. Analysis of the IgM and IgG Reibergrams showed that they had a disturbed blood-CSF barrier and intrathecal total-IgM synthesis in the absence of intrathecal total-IgG synthesis, which also supports the diagnosis of an early LNB. In our larger validation study (T. van Gorkom, W. Voet, G. H. J. van Arkel, M. Heron, S. F. T. Thijsen, and K. Kremer, unpublished data), in which we validated five commercial LNB assays, these three patients had intrathecal *Borrelia*-specific antibody synthesis detected by two or more of the LNB assays under investigation. This suggests that the sensitivity of the IDEIA LNB assay used in the current study is lower in the early stages of LNB, as has been reported previously [11, 54]. Four (50.0%) of the eight possible-LNB patients with pleocytosis had a negative CSF CXCL13 result in both assays. These four patients had a rather short duration of symptoms (range, 5 to 39 days), and three of them also had relatively low CSF cell counts (range, 6 to 21 cells/μl). A low CSF cell count and a negative CSF CXCL13 result among LNB patients has also been observed by Markowicz et al. [33]. The fourth patient for whom the CSF cell count was much higher (i.e.,

207 cells/µl) had elevated CSF CXCL13 levels in both assays, but these levels were below the cutoff values of the two assays. Since another diagnosis was never made for these four patients and they responded well to antibiotic treatment, LNB was the most likely diagnosis. The last patient who was classified as a possible-LNB patient, and who had a negative CSF CXCL13 result in both assays, had intrathecal *Borrelia*-specific antibody synthesis and a normal CSF cell count. This patient reported symptoms for more than 6 months and therefore might have had LNB previously. Others have reported similar cases with a long symptom duration and high levels of intrathecally produced *Borrelia*-specific IgG and have speculated that the inflammation might have been resolved by the time of the LP, as shown by normal CSF cell counts and CSF CXCL13 levels, and that this probably reflects the natural course of the disease [14, 55].

In this study, we also found elevated CXCL13 levels in the CSF of patients with other infectious CNS diseases, such as neurosyphilis and tuberculous meningitis, which has been reported before [21, 26, 27, 30]. Elevated CSF CXCL13 levels have also been found in patients with HIV, *Cryptococcus neoformans* meningitis, congenital toxoplasmosis [21, 28], viral encephalitis [32], viral meningitis (e.g., varicella zoster virus, herpes simplex virus, and tick-borne encephalitis virus [25, 28, 33]), multiple sclerosis [19, 28, 34], and CNS lymphoma [26, 35]. This underlines that CSF CXCL13 can be a marker for infection and/or inflammation of the CNS and not a specific marker for LNB. This prompted us to investigate the association between the CSF CXCL13 levels and the classification according to Reiber [45] based on the blood-CSF barrier functionality and the presence of intrathecal total-antibody synthesis among LNB patients. Both a dysfunctional blood-CSF barrier and intrathecal total-IgM synthesis are often found among patients with LNB [56-58]. In our study, all patients with a dysfunctional blood-CSF barrier and proof of intrathecal total-antibody synthesis (Reibergram area 3 [i.e., group 3]) had positive CSF CXCL13 results in both assays. The majority (8/9; 88.9%) of them either had definite LNB (n = 5) or possible LNB (n = 3). These results indicate a clear association between elevated CSF CXCL13 levels, a dysfunctional blood-CSF barrier, and intrathecal total-antibody synthesis among LNB patients. The three possible-LNB patients lacked intrathecal *Borrelia*-specific antibody synthesis, and this emphasizes the added value of using Reibergrams in the early diagnosis of LNB [56]. Nevertheless, positive CSF CXCL13 results in both assays were also found in a definite-LNB patient who had a normal blood-CSF barrier in the absence of intrathecal total-antibody synthesis (Reibergram area 1 [i.e., group 1]) and in two patients (one diagnosed with definite LNB and the other with tuberculous meningitis) who had a dysfunctional blood-CSF barrier and no proof of intrathecal total-antibody synthesis (Reibergram area 2 [i.e., group 2]).

Although the positivity rates of the CXCL13 assays used in this study gave comparable results among the three EFNS groups, the CXCL13 ELISA resulted in lower CXCL13 levels in the CSF than the *recom*Bead CXCL13 assay. This is underlined by the differences between the cutoff values of the two assays, 85.9 pg/ml for the CXCL13 ELISA and 252.2 pg/ml for the *recom*Bead CXCL13 assay. This difference in absolute CSF CXCL13 levels observed between the two assays could have various explanations. The assays are based on different platforms, and different calculation methods were used to assess the CSF CXCL13 concentrations. In the CXCL13 ELISA, a standard curve is calculated in each run based on seven standards with different concentrations [44]. In contrast, the *recom*Bead CXCL13 assay uses a one-point quantification by the inclusion of a calibrator in each run which is compared to the batch-dependent 4-PL coordinates of the standard curve stored in the *recom*Quant evaluation software [41]. The use of different capture and/or detection antibodies could also explain the differences found [59]. Some of these issues have previously been debated by Henningsson et al. and Markowicz et al., both of whom also reported different absolute amounts of CSF CXCL13 for the two assays [33, 36]. Henningsson et al. found lower cutoff values than we did (56 pg/ml for the CXCL13 ELISA and 158 pg/ml for the *recom*Bead CXCL13 assay) [36]. Similarly, Markowicz et al. also found a lower cutoff value (131

pg/ml) for the *recom*Bead CXCL13 assay [33]. The difference between these two studies and our study mainly consists of the study set-up and, consequently, the groups used for the ROC curve analyses. Differences in sample handling, age, and gender could also explain the differences in the CSF CXCL13 cutoff values between the various studies, as has been suggested by Rupprecht et al. [39]. The broad range of cutoff values found in the various studies underline the importance of determining a setting-specific cutoff value, irrespective of the assay, as absolute levels differ between different assays, as well as between identical assays validated by different study groups. Also, more research should be done to assess the cause of these differences in absolute levels of CSF CXCL13. These differences are intriguing and raise the question of whether an international reference standard could be defined.

This study had some limitations. First, for some CSF samples the CXCL13 concentration exceeded the upper measurement limit. These samples were not diluted any further to determine the final CSF CXCL13 concentration. Consequently, and also given the limited number of patients, it was not possible to investigate the correlation between CSF CXCL13 levels and symptom duration. Nevertheless, Henningsson et al. also reported a weak positive correlation between higher CSF CXCL13 and a longer symptom duration [60]. In contrast, others found either a negative correlation or no correlation at all [25, 31, 61]. Since a positive correlation between CSF CXCL13 levels and symptom duration could be very interesting, as this stresses the role of CSF CXCL13 as an early marker for infection, this should be investigated further in a prospective study. Another limitation was the retrospective design of the study. As a consequence, data could be incomplete, which could have had an effect on the results, e.g., if data on antibiotic treatment are lacking. Antibiotic treatment can abrogate antibody production, as well as CSF CXCL13 production, and thus give rise to false-negative results if given prior to the LP, consequently leading to an underestimation of the sensitivity of the assay under investigation [20, 61, 62]. In this study, all definite-LNB and possible-LNB patients were treated for LNB according to the Dutch guidelines for LB [53], which had started after the LP was performed. Even though the electronic patient files of the 156 patients included in this study were analyzed extensively prior to the start of the study, information regarding antibiotic treatment could have been lacking for patients who received antibiotics not related to a visit in our hospital in the weeks preceding the LP. Most patients from whom a CSF sample was taken were excluded in this study due to the absence of a serum sample, insufficient amounts of CSF and/or serum, hemolytic CSF, or because they were treated intravenously with IgG [40, 41]. We do not expect this would have led to a bias in our study. The percentage of patients with *Borrelia*-specific serum antibodies among the non-LNB patients (22.0%) (Table 1) exceeded that of the general population in the Netherlands (4 to 8%) [53], which suggests that patients with a history of a tick bite and/or previously detected *Borrelia*-specific serum antibodies are more often subjected to an LP. We believe this represents the clinical practice and not a bias in our study. Another limitation was the unknown number of freeze-thaw steps to which the CSF samples had been subjected (all CSF samples were stored at either -20°C or -80°C); however, the number of freeze-thaw steps was expected to be limited. We therefore do not believe this has negatively influenced the results in this study, as CXCL13 is stable when subjected to repeated freeze-thaw cycles, as shown by Hytonen et al. (for up to a maximum of five freeze-thaw cycles) [25] and by personal experience (for up to four freeze-thaw cycles investigated) (data not shown). Rupprecht et al., however, reported a negative correlation between CSF CXCL13 levels and the number of freeze-thaw cycles but does not mention the number of freeze-thaw cycles that was investigated [39]. We did find a negative correlation between CSF CXCL13 levels and storage time (for up to 3 days at +4°C) before freezing at -20°C (data not shown), which was also reported by Rupprecht et al. [39]. Prolonged storage at either -20°C or -80°C could also have had an effect on the CSF CXCL13 levels, although studies report differently on this issue [26, 28, 39]. Another limitation of this study was the limited numbers of definite-LNB and possible-LNB patients who were diagnosed in our hospital. By choosing a cross-

sectional design that comprised almost 3 years (from August 2013 to June 2016), the expected number of LNB patients to be included was 15. This number is based on the annual incidence rate of LNB in the Netherlands in 2010 (i.e., 2.6 per 100,000 inhabitants), the number of general hospitals in the Netherlands (*n* = 84) in that year, and the Dutch population in 2010 (16,600,000) [4, 63, 64]. In the current study, however, only nine LNB patients (three with definite and six with possible LNB) fulfilled the inclusion criteria. To reach the expected number of 15 LNB patients for the predefined study period, we added six LNB patients from outside this period. Preferably, the study period would have been extended, but this would have cost much more time and testing, as this would also have resulted in a much larger non-LNB group. Since we do not think the composition of the non-LNB group would be any different, we believe the cross-sectional design of the study still holds. Ideally, the results of this study should be confirmed in a prospective design aiming at inclusion of more LNB patients and controls with other proven non-LNB diseases.

As discussed above, this study was based on a cross-sectional design, which can be considered a strength of this study as discussed by Rupprecht et al. and Leeflang et al. [38, 39]. Another strength of this study is the use of a well-defined study population. Due to the nature of our study, possible-LNB patients were included for whom insight into the added value of using CSF CXCL13 as a diagnostic assay is of particular interest, especially for those with clinical symptoms suggestive of LNB and pleocytosis, as was discussed by Rupprecht et al. [39].

In conclusion, this retrospective, cross-sectional study confirms that determining the CSF CXCL13 levels can aid in the diagnosis of LNB. None of the definite-LNB patients had a negative CSF CXCL13 result in both assays. Furthermore, we show the added value of this marker for the group of possible-LNB patients who have pleocytosis without intrathecally produced *Borrelia*-specific

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SUPPLEMENTAL MATERIAL

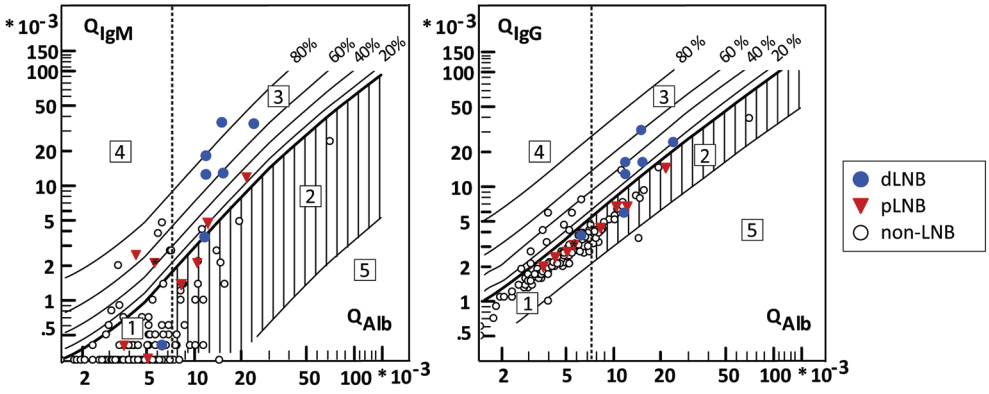

Fig. S1. CSF/serum quotients for the 156 patients included in the study visualized in CSF/serum quotient diagrams (Reibergrams) for IgM (left) and IgG (right) including the five areas as described by Reiber [1]. The black dashed vertical lines represent the average age-dependent reference limit of Q Alb (Q_{lim} Alb) among the 156 patients, which was $7.5*10^{-3}$ (data not shown). The x-axes show the CSF/serum quotients for albumin (Q Alb), and the y-axes show the CSF/serum quotients for total IgM (Q IgM) and total IgG (Q IgG), respectively. Definite Lyme neuroborreliosis (dLNB) patients are indicated in blue dots, possible LNB (pLNB) patients in red triangles, and non-Lyme neuroborreliosis (non-LNB) patients in open circles. One of the eight possible LNB patients was a child for whom the Q_{lim} Alb was $4.7*10^{-3}$ (red triangle with black asterisk) and for whom a dysfunctional blood-CSF barrier was established; however, in the Reibergrams this patient is located to the left of the average Q_{lim} Alb. Two non-LNB patients were located slightly below the lower reference limit of Q IgG, however, they were classified to either the first group (normal blood-CSF barrier and no proof of intrathecal IgG synthesis, or the second group (dysfunctional blood-CSF barrier and no proof of intrathecal IgG synthesis).

Table S2. Classification of the 156 patients according to their position in the IgM and IgG Reibergram and their overall Reibergram classification.

	Area in IgG Reibergram				
Area in IgM Reibergram	**RG1**	**RG2**	**RG3**	**RG4**	**Total**
RG1	107[a]			7[b]	114
RG2		25[c]			25
RG3		3[d]	6[e]		9
RG4	7[f]			1[g]	8
Total	114	28	6	8	156

All patients were classified according to their position in the IgM and IgG Reibergram as described by Reiber [1], and as is shown in supplemental Figure S1. Following this description, patients with a Q IgM (or Q IgG) located in the first area (Reibergram group 1; RG1) had a normal blood-CSF barrier and absence of intrathecally produced total IgM (or IgG). Patients with a Q IgM (or Q IgG) located in the second area (Reibergram group 2; RG2) had a dysfunctional blood-CSF barrier without proof of intrathecal total IgM (or IgG) synthesis. Patients with a Q IgM (or Q IgG) located in the third area (Reibergram group 3; RG3) had a dysfunctional blood-CSF barrier with proof of intrathecal total IgM (or IgG) synthesis. Patients with a Q IgM (or Q IgG) located in the fourth area (Reibergram group 4; RG4) had a normal blood-CSF barrier and proof of intrathecal total IgM (or IgG) synthesis. Based on the combined IgM and IgG Reibergrams, the overall Reibergram classification was as follows: RG1: 107 patients (white box), RG2: 25 patients (light grey box), RG3, nine patients (dark grey boxes), and RG4: 15 patients (black boxes).

a. One definite, two possible, and 104 non-LNB patients.
b. All non-LNB patients.
c. One definite, two possible, and 22 non-LNB patients.
d. All possible LNB patients.
e. Five definite and one non-LNB patient.
f. One possible and six non-LNB patients.
g. One non-LNB patient.

REFERENCE OF SUPPLEMENTAL MATERIAL

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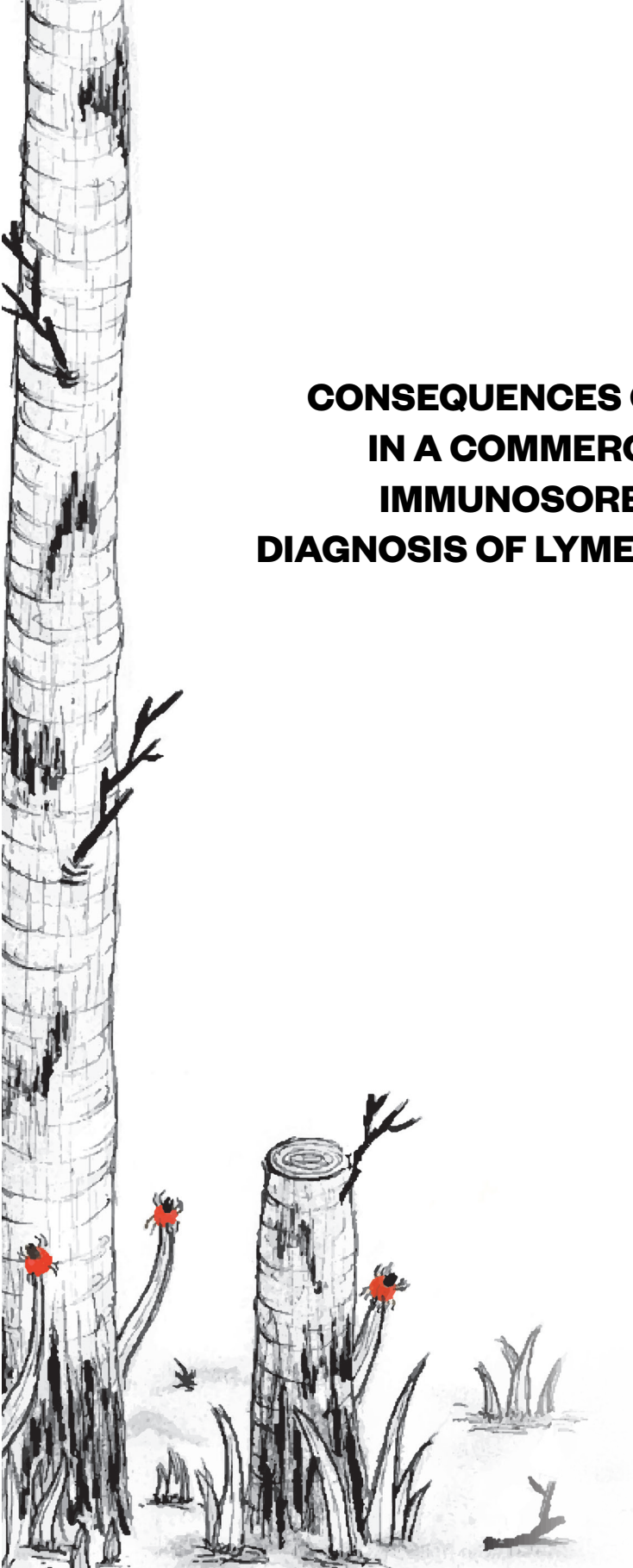
CONSEQUENCES OF THE EDGE EFFECT IN A COMMERCIAL ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DIAGNOSIS OF LYME NEUROBORRELIOSIS



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ABSTRACT

The diagnosis of Lyme neuroborreliosis (LNB) is based on neurological symptoms, cerebrospinal fluid (CSF) pleocytosis, and intrathecally produced *Borrelia*-specific antibodies. In most cases, the presence of intrathecally produced *Borrelia*-specific antibodies is determined by using an enzyme-linked immunosorbent assay (ELISA). The edge effect is a known phenomenon in ELISAs and can negatively influence the assay reproducibility and repeatability, as well as index calculations of sample pairs which are tested in the same run. For LNB diagnostics, an index calculation is used for which the relative amounts of *Borrelia*-specific antibodies in CSF and serum are measured to calculate a CSF/serum quotient, which is needed to calculate the *Borrelia*-specific antibody index (AI). The presence of an edge effect in an ELISA used for LNB diagnostics may thus have implications. In this study, we investigated the intra-assay variation of the commercial Enzygnost Lyme link VlsE/IgG ELISA used for LNB diagnostics and showed the presence of an edge effect. Minor adaptations in the ELISA protocol decreased this effect. The adapted protocol was subsequently used to test 149 CSF-serum pairs of consecutive patients received in a routine diagnostic laboratory. By simulation, we showed that, if the standard protocol would have been used, then the edge effect for this study population could have resulted in 15 (10.1%) false-pathological and two (1.3%) false-normal *Borrelia*-specific IgG AIs. Thus, the observed edge effect can lead to inaccurate LNB diagnoses. Our study underlines that the edge effect should be investigated when ELISAs are implemented in routine diagnostics, as this phenomenon can occur in any ELISA.

KEYWORDS

intra-assay variation, edge effect, ELISA, index calculation, Lyme neuroborreliosis

Lyme borreliosis is a tick-borne disease. In the Netherlands, the incidence of Lyme borreliosis has quadrupled in the last 2 decades, with an estimated number of 25,500 cases of erythema migrans and 1,500 cases of disseminated disease in 2017 (https://www.rivm.nl/en/Documents_and_publications/Common_and_Present/Newsmessages/2018/Lyme_disease_cases_have_quadrupled). The annual incidence rate of Lyme neuroborreliosis (LNB), a manifestation of the nervous system, was 2.6 (95% confidence interval [CI], 2.4 to 2.8) per 100,000 Dutch inhabitants in 2010 [1]. Clinical symptoms of LNB include radiculopathy, cranial neuropathy (often facial palsy), paresthesia, meningitis, and meningoencephalitis. As prompt treatment has proven effective, an accurate diagnosis of these severe forms of Lyme borreliosis is pivotal [2].

The laboratory diagnosis of LNB includes the detection of intrathecally produced *Borrelia*-specific antibodies. Intrathecal antibody production is established by the measurement of the number of *Borrelia*-specific antibodies in cerebrospinal fluid (CSF) and serum followed by an antibody index (AI) calculation as described by Reiber and Peter [3, 4]. *Borrelia*-specific antibodies in CSF and serum should be measured in the same run to exclude interassay variation and, hence, ensure optimal accuracy. Across the testing plate used for antibody detection, variations can occur that are most pronounced at the edges of an ELISA plate. These variations are shown by a difference in the optical densities (OD) between inner (lower OD) and outer (higher OD) wells of an ELISA plate and are referred to as the "edge effect" [5-7]. The presence of intra-assay variations such as the edge effect could potentially lead to false-positive or false-negative results and should therefore be investigated when ELISAs are used in routine diagnostics [6, 8, 9]. In this study, we investigated the performance of a commercial ELISA used for the diagnosis of LNB and observed an edge effect. We analyzed the consequences of this effect on the AI calculation and subsequent impact on LNB diagnostics through simulation. We also show some minor adaptations to the protocol which can lower the well variation across the plate.

MATERIALS AND METHODS

VERIFICATION OF THE ENZYGNOST IGG ELISA

In a first experiment, the intra-assay variation of the commercial Enzygnost Lyme link VlsE/IgG ELISA (Siemens Healthcare Diagnostics, Marburg, Germany), here referred to as the Enzygnost IgG ELISA, was investigated. The Enzygnost IgG ELISA consists of a 96-well plate holder harboring 12 strips, each consisting of 8 wells coated with a mixture of deactivated *Borrelia afzelii* antigens (PKo isolate) and recombinant VlsE from *B. afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto (10). In this experiment, a total of 4 test strips (32 wells) were used, which were placed in the first 4 columns of a 96-well plate holder. Thirty wells were tested with a single dilution (1:231) of the positive kit control (human serum with specific IgG to *B. burgdorferi*), which was subsequently divided over 30 wells. The remaining wells were tested with the negative kit control (human serum without antibodies to *B. burgdorferi*) (Table 1A).

In a second experiment, the intra-assay variation of the Enzygnost IgG ELISA was further investigated. The second experiment was identical to the first except for an additional strip filled with distilled water, which was placed in the fifth column of the 96-well plate holder (Table 1B). Both experiments were performed according to the instructions of the manufacturer by using a Dynex DS2 automated ELISA instrument (Dynex Technologies, Chantilly, VA, USA) [10].

In a third experiment, the intra-assay variation of the Enzygnost IgG ELISA was assessed by using a different ELISA robot. This experiment resembled the first; however, a single dilution (1:231) of the positive kit control was tested in 6 strips (48 wells) that were placed in the first 6 columns of a 96-well plate holder (Table 1C). The negative kit control was not included in this experiment. The

ELISA was performed according to the instructions of the manufacturer by using the BEP 2000 system (Siemens Healthcare Diagnostics) [10].

Table 1. Overview of the setup of the three experiments performed in this study to investigate the intra-assay variation of the Enzygnost IgG ELISA[a]

A	Columns				B	Columns					C	Columns					
Rows	1	2	3	4	Rows	1	2	3	4	5	Rows	1	2	3	4	5	6
1	NC	PC	PC	PC	A	NC	PC	PC	PC	DW	A	PC	PC	PC	PC	PC	PC
2	PC	PC	PC	PC	B	PC	PC	PC	PC	DW	B	PC	PC	PC	PC	PC	PC
3	PC	PC	PC	PC	C	PC	PC	PC	PC	DW	C	PC	PC	PC	PC	PC	PC
4	PC	PC	PC	PC	D	PC	PC	PC	PC	DW	D	PC	PC	PC	PC	PC	PC
5	PC	PC	PC	PC	E	PC	PC	PC	PC	DW	E	PC	PC	PC	PC	PC	PC
6	PC	PC	PC	PC	F	PC	PC	PC	PC	DW	F	PC	PC	PC	PC	PC	PC
7	PC	PC	PC	NC	G	PC	PC	PC	NC	DW	G	PC	PC	PC	PC	PC	PC
8	PC	PC	PC	PC	H	PC	PC	PC	PC	DW	H	PC	PC	PC	PC	PC	PC

Panel A shows, in total, 32 wells that were tested in 4 strips, which were placed in the first 4 columns of a 96-well plate holder on an automated DS2 instrument (Dynex Technologies). A single dilution (1:231) of the positive kit control (PC) was tested in 30 wells, and the negative kit control (NC) was tested in 2 wells. Panel B is like panel A; however, an additional strip filled with distilled water (DW) was placed in the fifth column of the 96-well plate holder. In panel C, a single dilution (1:231) of the PC was tested in 6 strips (48 wells), which were placed in the first 6 columns of a 96-well plate holder on an automated BEP 2000 system (Siemens Healthcare Diagnostics).

INTRA-ASSAY VARIATION AND STATISTICAL ANALYSES

To assess the intra-assay variation of the Enzygnost IgG ELISA, the intra-assay coefficients of variation (CV) of the OD values of the positive kit control were calculated by using the formula CV = (standard deviation of the OD values/mean OD value) x 100%. The CVs were calculated for each column, row, and plate. The negative kit control was not included in any of the analyses performed.

To further assess the performance of the assay, the median OD values of the columns were compared by using Friedman's test [11]. Subsequent pairwise comparisons were done by using the Nemenyi *post-hoc* test (https://CRAN.R-project.org/package=PMCMRplus). Raw *P* values of <0.05 were interpreted as statistically significant; however, to account for the multiple statistical analyses in this study, the Benjamini-Hochberg procedure (BH) was applied. Therefore, the false-discovery rate (FDR) was set at the level of 5.0%, and less than one false-positive result was allowed in the list of rejections [12]. For all statistical analyses, RStudio (version 1.3.959, 2009 to 2020; Boston, MA, USA) was used [13]. For construction of the figures, GraphPad Prism version 5.04 for Windows was used (GraphPad Software, San Diego, CA, USA).

STANDARD VERSUS ADAPTED ENZYGNOST IGG ELISA PROTOCOL

In the instruction manual of the manufacturer of the Enzygnost IgG ELISA, the following two standard protocols are described for the testing of CSF-serum pairs, which depends on the test system used: (i) for manual testing or testing with the automated BEP III system (Siemens Healthcare Diagnostics), each CSF-serum pair should be tested in consecutive wells, column-wise, starting with the serum sample; and (ii) for testing with the automated BEP 2000 system (Siemens Healthcare Diagnostics), all serum samples of the patients to be tested should be tested first in consecutive wells and column-wise, followed by all the corresponding CSF samples [14].

For use in our validation study (see next paragraph), the standard protocol was adapted in the following two ways before the patient samples were tested: (i) two strips filled with distilled water were added in each run, one located before the first strip, the other located after the last

strip; and (ii) all CSF-serum pairs were tested row-wise in consecutive wells. For all other steps, the standard protocol was used [14].

CRITERIA FOR THE CALCULATION OF THE *BORRELIA*-SPECIFIC CSF/SERUM IGG QUOTIENT AND THE *BORRELIA*-SPECIFIC IGG AI

In order to obtain the CSF and serum concentrations needed to calculate the *Borrelia*-specific CSF/serum IgG quotient, we followed the instructions of the manufacturer [10]. The raw OD values of the wells in each run were adjusted with a correction factor to correct for interassay variation. This correction factor was calculated for each run by using the following formula: correction factor = lot-specific OD value of the positive kit control/mean OD value of the positive kit control in each run. Subsequently, the corrected OD values were used to calculate the concentration (in units per milliliter) by using the α-method; however, only when the following two criteria were fulfilled: (i) the raw OD value should be 2.5 or lower, and (ii) the corrected OD value should be higher than that of the run-specific cutoff. This cutoff was based on the mean OD value of the negative kit control plus 0.150 [10].

According to the instructions of the manufacturer for LNB diagnostics, the *Borrelia*-specific CSF/serum IgG quotient may be calculated when the following two criteria are met: (i) the CSF and serum concentration must lie within the measurement range, and (ii) the concentration quotient must lie between 1.0 and 3.0 [14]. The concentration quotient is calculated by dividing the higher concentration by the lower concentration before correction with the corresponding dilution factor. The lower concentration limit of the measurement range is run dependent and defined by the concentration of the run-specific cutoff. The upper limit of the measurement range is run independent and set at 300 U/ml. If the initial dilution of the CSF and/or serum results in a concentration that exceeds 300 U/ml, then further dilutions are needed before the *Borrelia*-specific CSF/serum IgG quotient may be calculated. In case multiple dilutions are tested, the CSF-serum pair that best fits the criteria for calculation of the *Borrelia*-specific CSF/serum IgG quotient is chosen. The *Borrelia*-specific CSF/serum IgG quotient is calculated by dividing the CSF concentration by the serum concentration after correction with the dilution factor. The *Borrelia*-specific IgG AI is calculated according to Reiber and Peter [4]. AI values between 0.5 and 1.49 are considered normal, AI values ≥ 1.5 are considered pathological, and AI values < 0.5 indicate a measurement error or a sample mix-up [14].

STUDY POPULATION USED TO INVESTIGATE THE POSSIBLE CONSEQUENCES OF USING THE STANDARD PROTOCOL FOR LNB DIAGNOSTICS

To investigate the possible consequences of using the standard protocol for the measurement of *Borrelia*-specific IgG in CSF-serum pairs and subsequent *Borrelia*-specific IgG AI calculation, we used the Enzygnost IgG ELISA results that were part of a larger validation study (T. van Gorkom, W. Voet, G. H. J. van Arkel, M. Heron, S. F. T. Thijsen, and K. Kremer, unpublished data). In this validation study, all consecutive patients were included from whom a CSF-serum pair, taken less than 24 h apart, was sent to the laboratory of the Diakonessenhuis Hospital, Utrecht, the Netherlands, in the period between August 2013 and June 2016. Until the start of this study in 2017, leftover material from these patients (both CSF and serum) had been stored at -20°C and/or -80°C. Prior to the start of this study, all samples had been freeze-thawed once to aliquot for use in multiple assays for the larger validation study and were stored at -20°C until use. Patients for whom an insufficient amount of CSF and/or serum was available were excluded. In total, 156 consecutive patients were included in this study, and the CSF and serum pairs of 149 of them were tested by using the Enzygnost IgG ELISA. Seventy-one (47.7%) of the 149 patients were male, and their mean age was 52.0 years (95% CI: 49.4 to 54.7). Twenty-nine (19.5%) patients had pleocytosis (≥5 leucocytes/µl) based on the CSF cell counts that had been determined at the time of the lumbar puncture in the past.

All CSF and serum pairs used in this study were anonymized and, according to the rules of our hospital, approval of the local ethics committee was not necessary as the main goal of our study was assay validation for which leftover material could be used. We did, however, obtain approval for this study from the hospital board.

CONSTRUCTION OF SIMULATED ENZYGNOST IGG ELISA RESULTS BY USING TWO SCENARIOS

Intra-assay variation by using the standard protocol of the manufacturer can influence the ELISA results and, hence, the *Borrelia*-specific IgG AI. For instance, if the CSF of a CSF-serum pair is tested in a well located in a certain position in the plate that structurally leads to higher OD values than a well located elsewhere in the same plate, then this could result in a higher concentration, a higher *Borrelia*-specific CSF/serum IgG quotient, and a higher *Borrelia*-specific IgG AI. This could thus also affect the interpretation of the AI. To gain insight into the consequences thereof, we used the Enzygnost IgG results of the 149 consecutive patients that were obtained by using the adapted protocol. In this adapted protocol, CSF and serum of a CSF-serum pair were tested in such a way to minimize the intra-assay variation of the standard protocol of the manufacturer. By using these Enzygnost IgG results, we constructed so-called simulated Enzygnost IgG results that could have been obtained when the standard protocol would have been used, and either CSF or serum of a CSF-serum pair was tested in a well that was subjected to the intra-assay variation. To construct these simulated Enzygnost IgG ELISA results, the raw OD values of either the CSF or the serum of the CSF-serum pairs (obtained by using the adapted protocol) were subjected and adjusted to the intra-assay variation found in the first experiment. Therefore, we determined a factor that reflected the magnitude of the intra-assay variation. As the intra-assay variation was most prevalent between the columns in the first experiment, we based the factor on the largest difference in median (raw) OD value of the positive kit control found between any of the two columns. This factor was calculated by dividing the highest median OD value of any of the four columns by the lowest median OD value of any of the four columns. By using this factor, two scenarios were investigated. In the first scenario, the CSF of a CSF-serum pair was simulated as if it had been tested by using the standard protocol in a well in the column that displayed the highest median OD value. Therefore, the raw OD value of the CSF, obtained by using the adapted protocol, was adjusted through multiplication by the factor reflecting the magnitude of the intra-assay variation. In this scenario, the raw OD value of the serum, obtained by using the adapted protocol, was not adjusted. In the second scenario, the serum of a CSF-serum pair was simulated as if it had been tested by using the standard protocol in a well in the column that displayed the highest median OD value. Therefore, the raw OD value of the serum, obtained by using the adapted protocol, was multiplied by the factor reflecting the magnitude of the intra-assay variation. In this scenario, the raw OD value of the CSF, obtained by using the adapted protocol, was not adjusted. Subsequently, in both scenarios, the raw OD values were corrected, the concentrations were calculated, the simulated *Borrelia*-specific CSF/serum IgG quotients were determined, and, hence, the simulated *Borrelia*-specific IgG AIs were calculated according to the instructions of the manufacturer (and also described above) [10, 14]. By using the simulated *Borrelia*-specific IgG AIs, the impact of the intra-assay variation was assessed.

RESULTS

THE PERFORMANCE OF THE ENZYGNOST IGG ELISA BASED ON THE MEASURED OD VALUES

To visualize the intra-assay variation of the Enzygnost IgG ELISA, heat maps of the OD values obtained in the first and second experiment were constructed, which showed that higher OD values were found in the outer wells than those in the inner wells (Table 2A and B). This phenomenon is also described as the edge effect [5-7]. In the first experiment, an overall mean CV of 8.9% was found among the wells filled with the positive kit control. More variation was found between the OD values in the wells in rows than between the OD values in the wells in columns (mean CV, 8.4% and 4.0%, respectively; Table 2A). The addition of a strip filled with distilled water adjacent to the last test strip in the second experiment, thereby decreasing the number of outer wells, resulted in a lower overall mean CV of 4.4% (Table 2B). This also resulted in a lower variation between the OD values in the wells in rows, which was now comparable to the variation between the OD values in the wells in columns (mean CV, 3.2% and 3.8%, respectively; Table 2B).

Further analyses showed that the OD values in the wells in the outer columns (columns 1 and 4 in the first experiment and column 1 in the second experiment) were higher than those in the wells in the inner columns (Table 2A and B; Fig. 1A and B). In the first experiment, the OD values in the wells in both the first and the fourth (both outer) columns were significantly higher than those in the third (inner) column (Fig. 1A). In the second experiment, the OD values in the wells in the first (outer) column were significantly higher than those in the fourth (now inner) column (Fig. 1B). By exclusion of the wells in the first column, only wells of similar location, either all located outside (first and last row) or all located inside (rows 2 to 7), were compared. This resulted in a further decrease of the variation found between the OD values in the wells in rows (mean CV, 1.3%) (Table 2B).

The third experiment was similar to the first experiment except for the use of a different automated ELISA instrument and the number of wells (48 instead of 30) filled with the positive kit control (Table 1C). Again, the heat map of the OD values showed that intra-assay variation was present across the wells tested (Table 2C). The variation was slightly different than that found in the first experiment. Still, an edge effect was observed, as higher OD values were found in the outer wells (sixth column and first row) than in the inner wells (Table 2C). In contrast to what was found in the first and second experiments, the OD values were lowest in the wells in the first column and increased from left to right (Fig. 1C). Thus, the OD values in the wells in the sixth column were the highest, which was significant compared to those in the first and second column (Fig. 1C). The OD values in the wells in the fifth column were also significantly higher than those in the first column.

Table 2. Heat maps and coefficients of variation (CV) of the optical density (OD) values of the positive kit control for the three experiments performed in this study[a]

A

	Columns				Row statistics	
Rows	**1**	**2**	**3**	**4**	**CV (%)**	**Mean CV (%)**
1	NC	0.843	0.819	0.975	9.6	8.4
2	0.793	0.737	0.725	0.886	9.3	
3	0.800	0.734	0.723	0.855	7.9	
4	0.815	0.759	0.754	0.905	8.7	
5	0.808	0.757	0.745	0.891	8.3	
6	0.780	0.752	0.744	0.898	9.0	
7	0.765	0.742	0.725	NC	2.7	
8	0.794	0.773	0.763	0.972	11.9	
Column statistics						**Overall statistics**
CV (%)	2.1	4.6	4.2	4.9		8.9
Mean CV (%)			4.0			

B

	Columns					Row statistics of columns 1 to 4		Row statistics of columns 2 to 4	
Rows	**1**	**2**	**3**	**4**	**5**	**CV (%)**	**Mean CV (%)**	**CV (%)**	**Mean CV (%)**
1	NC	0.686	0.678	0.671	DW	1.1	3.2	1.1	1.3
2	0.641	0.596	0.598	0.578	DW	4.4		1.9	
3	0.650	0.619	0.608	0.606	DW	3.3		1.1	
4	0.666	0.619	0.600	0.595	DW	5.2		2.1	
5	0.660	0.612	0.623	0.606	DW	3.9		1.4	
6	0.653	0.615	0.606	0.606	DW	3.6		0.9	
7	0.630	0.633	0.617	NC	DW	1.4		1.8	
8	0.661	0.625	0.621	0.620	DW	3.1		0.4	
Column statistics							**Overall statistics**		**Overall statistics**
CV (%)	1.9	4.3	4.1	4.8			4.4		4.3
Mean CV (%)			3.8						

C

	Columns						Row statistics	
Rows	**1**	**2**	**3**	**4**	**5**	**6**	**CV (%)**	**CV (%)**
1	1.191	1.194	1.205	1.198	1.215	1.243	1.6	4.8
2	1.092	1.095	1.102	1.121	1.109	1.198	3.6	
3	1.04	1.056	1.077	1.085	1.09	1.201	5.2	
4	1.047	1.083	1.084	1.082	1.085	1.2	4.8	
5	1.011	1.042	1.079	1.048	1.079	1.185	5.6	
6	1.012	1.046	1.05	1.047	1.074	1.188	5.7	
7	0.969	1.014	1.005	1.017	1.058	1.157	6.3	
8	1.025	1.073	1.097	1.1	1.14	1.202	5.4	
Column statistics								**Overall statistics**
CV (%)	6.4	5.0	5.2	5.1	4.6	2.0		6.3
Mean CV(%)				4.7				

a. The constructed heat maps show the levels of the OD values of the positive kit control in all wells tested, from low (blue) to high (orange). Panel A shows a heat map and CV of the positive kit control of which a single dilution (1:231) was tested in 30 wells of 4 strips that were placed in the first 4 columns of a 96-well plate holder on an automated DS2 instrument (Dynex Technologies). Panel B is like panel A; however, an additional strip filled with distilled water was placed in the fifth column of the 96-well plate holder. Panel C is a heat map and CV of the positive kit control of which a single dilution (1:231) was tested in 48 wells of six strips that were placed in the first six columns of a 96-well plate holder on an automated BEP 2000 system (Siemens Healthcare Diagnostics). CV, coefficient of variation; NC, negative control; DW, distilled water.

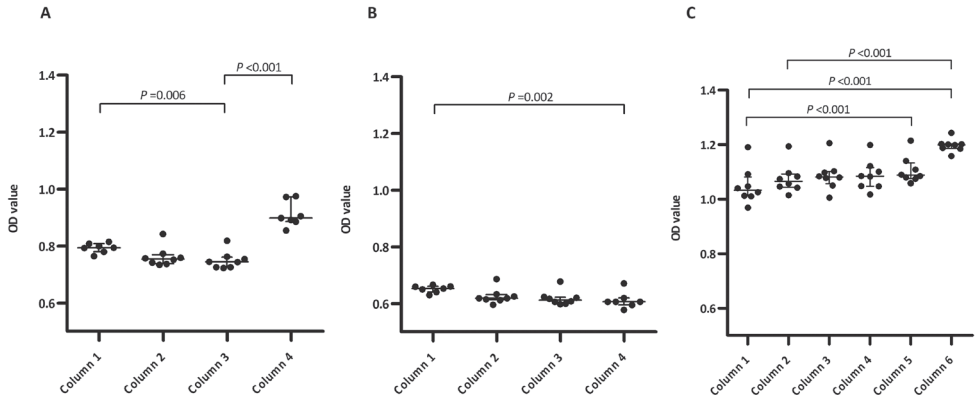

Fig. 1. Scatter dot plots of the optical density (OD) values of the positive kit control in the three experiments. The horizontal lines indicate the median OD values, and the whiskers represent the interquartile ranges (IQR; first and third quartiles). The displayed *P* values are raw *P* values that were found to be significant after applying the Benjamini-Hochberg procedure to account for the multiple comparisons in this study. (A) Scatter dot plots of the OD values of the positive kit control of which a single dilution (1:231) was tested in 30 wells of 4 strips that were placed in the first 4 columns of a 96-well plate holder on an automated DS2 instrument (Dynex Technologies). (B) Like panel A, however, with an additional strip filled with distilled water placed in the fifth column of the 96-well plate holder. (C) Scatter dot plots of the OD values of the positive kit control of which a single dilution (1:231) was tested in 48 wells in 6 strips that were placed in the first 6 columns of a 96-well plate holder on an automated BEP 2000 system (Siemens Healthcare Diagnostics).

BORRELIA-SPECIFIC IGG AI RESULTS BY USING THE ADAPTED ENZYGNOST IGG ELISA PROTOCOL

For LNB diagnostics, CSF and serum of a CSF-serum pair should be tested in the same run. In practice, by using the standard protocol of the manufacturer for the Enzygnost IgG ELISA, one of the two materials (either CSF or serum) could be tested in an outer well, and the other material could be tested in an inner well [14]. Consequently, the CSF-serum pair could be subjected to the edge effect that was observed in the first experiment. To minimize the consequences of the edge effect, we adapted the protocol of the Enzygnost IgG ELISA for use in our larger validation study. In this adapted protocol, strips filled with distilled water were placed in the first and last columns of the run, and CSF and serum from a single patient were tested in consecutive wells in the same row, as less variation was seen between wells of similar origin (both inner or both outer wells) as was shown in the second experiment. In total, CSF-serum pairs from 149 patients were tested in the Enzygnost IgG ELISA by using the adapted protocol. For 138 (92.6%) of the 149 patients, no proof for intrathecal *Borrelia*-specific IgG production was established. For 107 (71.8%) of the 149 patients, no detectable levels of *Borrelia*-specific IgG in the CSF were found, and, therefore, no AIs were calculated (Table 3 and Fig. 2A). Of those 107 patients, 18 (16.8%) had an elevated CSF cell count (≥5 leucocytes/µl; data not shown).

Table 3. Comparison of the *Borrelia*-specific IgG AI results of the 149 CSF-serum pairs by using the Enzygnost IgG ELISA results obtained by using the adapted protocol and by simulating the standard protocol

	AI results (no. [% of total] for protocol						
		Simulated standard protocol[b] in					
		Scenario 1			Scenario 2		
Results	**Adapted protocol[a]**	**Normal, not calculated[c]**	**Normal, AI < 1.5**	**Pathological, AI ≥ 1.5**	**Normal, not calculated[c]**	**Normal, AI < 1.5**	**Pathological, AI ≥ 1.5**
Normal, not calculated[c]	107 (71.8)	106		1	107		
Normal, 0.5 ≤ AI < 1.5	31[d,e,f] (20.8)		17	14[d,f,g,h]		31[d,e,f,h,i]	
Pathological, AI ≥ 1.5	11 (7.4)			11		2	9
Total	149	106 (71.1)	17 (11.4)	26 (17.4)	107 (71.8)	33 (22.1)	9 (6.0)

a. Adapted protocol shows the *Borrelia*-specific IgG AI results obtained when CSF and serum of a CSF-serum pair were tested in such a way that the edge effect was minimized (both outer or both inner wells).
b. Simulated standard protocol shows the *Borrelia*-specific IgG AI results obtained when either CSF or serum of a CSF-serum pair was subjected to the edge effect by investigating two scenarios. In the first scenario, the CSF was simulated to be tested in an outer well and the corresponding serum in an inner well. In the second scenario, the serum was simulated to be tested in an outer well and the corresponding CSF in an inner well. Simulation of testing in an outer well was done by multiplication of the raw OD value of either CSF (scenario 1) or serum (scenario 2) of a CSF-serum pair, obtained in the adapted protocol, with the edge-effect factor of 1.21 to reflect the magnitude of the edge effect.
c. Calculation of the AI was not possible due to the absence of *Borrelia*-specific antibodies in the CSF.
d. For one CSF-serum pair, the antibody concentrations for CSF and serum exceeded the upper measurement limit of 300 U/ml in the adapted protocol (323 U/ml and 310 U/ml, respectively), in the first scenario (558 U/ml and 310 U/ml, respectively), and in the second scenario (323 U/ml and 534 U/ml, respectively).
e. For one CSF-serum pair, the concentration quotient exceeded the upper concentration quotient limit of 3.0 in the standard protocol and in the second scenario (3.1 and 4.4, respectively; serum concentration > CSF concentration for both).
f. For one CSF-serum pair, the antibody concentration of the serum exceeded the upper measurement limit of 300 U/ml in the adapted protocol and in the first and second scenarios (302 U/ml, 302 U/ml, and 525 U/ml, respectively), and that of the CSF exceeded the upper measurement limit in the first scenario (339 U/ml).
g. For one CSF-serum pair, the concentration quotient in the first scenario was 3.3 (CSF concentration > serum concentration) and exceeded the upper concentration quotient limit of 3.0.
h. For one CSF-serum pair, the antibody concentration of the CSF in the first scenario (316 U/ml) exceeded the upper measurement limit of 300 U/ml. In the second scenario, the antibody concentration of the serum (454 U/ml) exceeded this limit.
i. For five CSF-serum pairs, the concentration quotients (range, 3.4 to 5.4; serum concentration > CSF concentration for all) exceeded the upper concentration quotient limit of 3.0.

For 42 (28.2%) of the 149 patients, an AI was calculated. The majority of them (31/42, 73.8%) had a normal *Borrelia*-specific IgG AI (Table 3 and Fig. 2A) of whom five (16.1%) had an elevated CSF cell count (≥5 leucocytes/µl; data not shown). Three (9.7%) of the 31 cases did not fulfill the criteria defined by the manufacturer required to calculate the *Borrelia*-specific CSF-serum IgG quotient and should have been repeated in a next ELISA run by using different dilutions (Table 3). For 11 (7.4%) of the 149 cases, a pathological *Borrelia*-specific IgG AI was found and thus proof of intrathecally produced *Borrelia*-specific IgG (Table 3 and Fig. 2A), and 6 (54.5%) of them had an elevated CSF cell count (≥5 leucocytes/µl; data not shown).

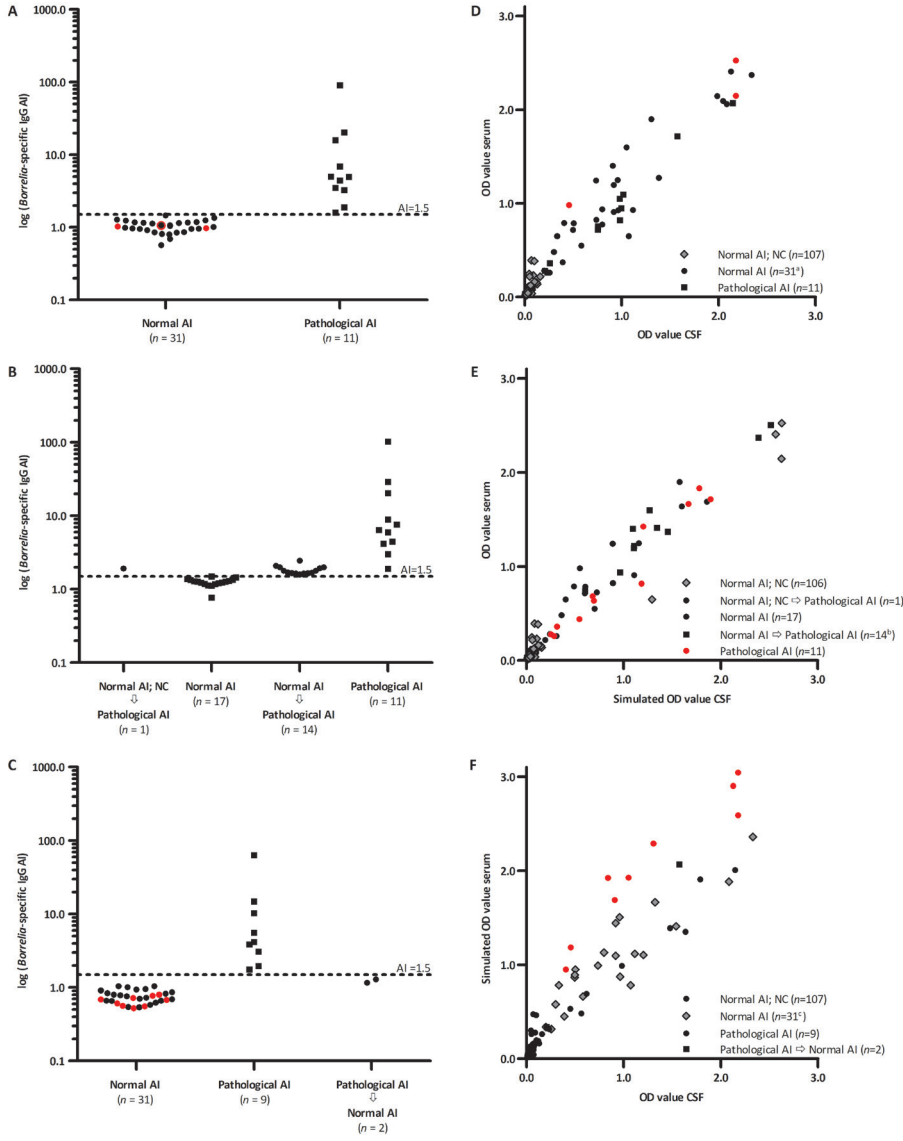

Fig. 2. Scatter dot plots of the (log-transformed) *Borrelia*-specific IgG antibody indices (AI) obtained by using the adapted protocol (n = 42) (A) or the simulated standard protocol based on either the first (n = 43) (B) or the second (n = 42) (C) scenario and the XY-plots of the (corrected) optical density (OD) values of the 149 CSF-serum pairs obtained by using the adapted protocol (D) or the simulated standard protocol based on either the first (E) or the second (F) scenario. The adapted protocol shows the results obtained when both CSF and serum of a CSF-serum pair were tested in outer wells, or in inners wells, to minimize the edge effect. The simulated standard protocol shows the results obtained when either CSF or serum of a CSF-serum pair was subjected to the edge effect by investigating two scenarios. In the first scenario, the CSF was simulated to be tested in an outer well and the corresponding serum in an inner well. In the second scenario, the serum was simulated to be tested in an outer well and the corresponding CSF in an inner well. Simulation of testing in an outer well was done by multiplication of the raw OD value of either CSF (scenario 1) or serum (scenario 2) of a CSF-serum pair, obtained in the adapted protocol, with the edge-effect factor of 1.21 to reflect the magnitude of the edge effect. All results are grouped by the results of the *Borrelia*-specific IgG AI (i.e., a normal AI for AIs that were not calculated [NC] due to undetectable levels of *Borrelia*-specific IgG in the CSF, a normal AI for AI values between 0.5 and 1.49, and a pathological AI for AI values ≥ 1.5). Red symbols show the CSF-serum pairs that did not fulfill the criteria for calculation of the *Borrelia*-specific CSF/serum IgG quotient and, hence, the *Borrelia*-specific IgG AI, which, in practice, would not have been calculated (see also Table 3).

CONSEQUENCES OF THE OBSERVED EDGE EFFECT IN THE ENZYGNOST IGG ELISA ON THE *BORRELIA*-SPECIFIC IGG AI DETERMINED BY SIMULATING THE ENZYGNOST IGG ELISA RESULTS AS IF THE STANDARD PROTOCOL HAD BEEN USED

By using the standard protocol, patient samples could be subjected to the edge effect. The magnitude of this edge effect was expressed by a factor which was calculated by dividing the highest median OD value (0.898; column 4) by the lowest median OD value (0.745; column 3) (Table 2A). This resulted in an "edge-effect factor" of 1.21, e.g., the OD values in the fourth column were 1.21 times higher than those in the third column. The presence of an edge effect could potentially lead to a false-pathological AI for those cases in which the CSF is tested in an outer well and the corresponding serum is tested in an inner well, as this results in a higher CSF/serum quotient and, thus, a higher AI. In contrast, a false-normal AI could be found when the serum is tested in an outer well and the corresponding CSF is tested in an inner well, as this will result in a lower CSF/serum quotient and, hence, a lower AI. Therefore, the edge-effect factor was used to create simulated Enzygnost IgG ELISA results in order to provide insight into the possible consequences for LNB diagnostics in case the standard protocol had been used. Two scenarios were investigated.

In the first scenario, in which the raw OD value of the CSF of a CSF-serum pair was multiplied by the edge-effect factor of 1.21, the number of *Borrelia*-specific IgG AI values greater than or equal to 1.5 increased from 11 to 26 (Table 3 and Fig. 2B). Thus, 15 CSF-serum pairs changed from a normal to a pathological *Borrelia*-specific IgG AI and were considered false pathological. All false-pathological AI values were located just above the AI cutoff value of 1.5 (Fig. 2B). For two (13.3%) of the 15 patients with a false-pathological AI, the CSF cell count was elevated (≥5 leucocytes/µl; data not shown). For four patients, all of whom had a false-pathological AI, the criteria for calculation of the *Borrelia*-specific CSF/serum IgG quotient were not fulfilled. Ideally, these CSF-serum pairs should be repeated in a next ELISA run by using different dilutions (Table 3).

In the second scenario, in which the raw OD value of the serum of a CSF-serum pair was multiplied by the edge-effect factor of 1.21, the number of *Borrelia*-specific IgG AI values greater than or equal to 1.5 decreased from 11 to 9 (Table 3 and Fig. 2C). Therefore, the results for two patients were considered to be false normal. Both false-normal AI values were located just below the AI cutoff value of 1.5 (Fig. 2C), and for both CSF samples, a normal cell count (<5 leucocytes/µl) was found (data not shown). By using the second scenario, the criteria for calculation of the *Borrelia*-specific CSF/serum IgG quotient were not fulfilled for nine patients (all of whom had a normal AI). Ideally, these CSF-serum pairs should be repeated in a next ELISA run by using different dilutions (Table 3).

ANALYSES OF THE CORRECTED OD VALUES AMONG THE 149 CSF-SERUM PAIRS OBTAINED BY USING THE ADAPTED PROTOCOL AND THOSE OBTAINED IN THE TWO SIMULATED SCENARIOS

The *Borrelia*-specific IgG AI does not provide insight into the OD values of the CSF and serum used to calculate the *Borrelia*-specific CSF/serum IgG quotient and the *Borrelia*-specific IgG AI. Yet this information is important for the interpretation of the AI, as a pathological AI can result from low (or high) OD values and might not indicate disease. The relationship between the OD values (plotted on the y-axis) and the (log-transformed) concentrations (plotted on the x-axis) is described by a sigmoid-shaped curve. When the OD values of both materials are either located in the lower left corner or the upper right corner of the curve, small differences between the OD values will result in larger differences between the concentrations, as is reflected by an almost horizontal line in the sigmoid-shaped curve in both corners. Thus, when the OD value of the serum is located in the lower left part of the curve on the horizontal line (and is lower than the OD value of the CSF), or when the OD value of the CSF is located in the upper right part of the

curve on the horizontal line (and is higher than the OD value of the serum), the difference in concentration will be larger, which will result in an increased CSF/serum quotient as well as an increased AI. Therefore, AIs should be interpreted with care.

The XY-plots that were constructed for each of the three protocols (i.e., the adapted protocol and the two scenarios used to simulate the standard protocol) show the distribution of the OD values of the 149 CSF-serum pairs (Fig. 2D to F). In each protocol, the CSF-serum pair was selected that best matched the criteria for calculation of the *Borrelia*-specific CSF/serum IgG quotient. Consequently, for 17 (40.5%) of the 42 patients for whom an AI was calculated by using the adapted protocol, a different set of dilutions was used in at least one of the two scenarios in which the standard protocol was simulated (data not shown). Analyses of the XY-plots showed that the distribution of the OD values of CSF and serum used for the index calculation were comparable between the three protocols (Fig. 2D to F). For those cases for which an AI had not been calculated in any of the three protocols, the OD values of CSF and serum were located in the lower left corner of the plots (Fig. 2D to 2F). The OD values of the CSF and serum for those patients for whom the calculated AI was normal were distributed across the whole range of OD values for both CSF and serum, irrespective of the protocol used (Fig. 2D to F). Similarly, the OD values of the patients who had a pathological AI were distributed across the whole range of OD values for both CSF and serum and covered the same OD range as those who had a normal AI. For the 14 patients with a normal AI when using the adapted protocol and a false-pathological AI in the first scenario, the OD values were also distributed across the whole range of OD values for both CSF and serum. For a single patient for whom the AI was not calculated by using the adapted protocol and in the second scenario, and who had a pathological AI in the first scenario, the OD values were relatively low (Fig. 2E).

DISCUSSION

The commercial Enzygnost Lyme Link VlsE/IgG ELISA can be used for the diagnosis of LNB by the detection of *Borrelia*-specific IgG in CSF-serum pairs [10, 14]. For LNB diagnostics, the results of the CSF-serum pairs are used to calculate a *Borrelia*-specific CSF/serum IgG quotient, after which a *Borrelia*-specific IgG AI is calculated according to Reiber and Peter [4]. The reliability of the ELISA results, however, can be affected by variations across the plate. In the current study, an edge effect was established for the Enzygnost IgG ELISA when run on a Dynex DS2 automated ELISA instrument (Dynex Technologies). The edge effect contributed to an overall mean CV of 8.9% in the OD values of a single dilution of the positive kit control. The edge effect was decreased by lowering the number of outer wells, which resulted in an overall mean CV of 4.3%. Similar results were obtained when the Enzygnost IgG ELISA was run on an automated BEP 2000 ELISA system (Siemens Healthcare Diagnostics).

In this study, we showed that the impact of the edge effect on CSF-serum pairs could be reduced by some minor adaptations to the standard protocol of the manufacturer [14], which, in this case, only applies for testing on the Dynex DS2 automated ELISA instrument (Dynex Technologies). For use in LNB diagnostics, the standard protocol of the Enzygnost IgG ELISA prescribes that CSF-serum pairs should be tested column-wise, either in consecutive wells or in wells placed farther apart. Consequently, one of the two materials could be located in an outer well and be subjected to the edge effect. In this study, we showed that when CSF-serum pairs are tested in wells of the same row (either both inside or both outside), less intra-assay variation is found. To ensure that only inner columns are used, a strip filled with distilled water should be placed before the first test strip and after the last test strip in a single run. Although we only tested the effect of placing a strip of distilled water in the last column, which can be seen as a limitation of this study,

we believe the same effect will occur when a strip filled with distilled water is placed in the first column, as was shown in the second experiment. In this experiment, less variation was seen by the removal of the OD values obtained in the wells in the first column. The only difference is that the wells in the first column were filled with the positive kit control instead of distilled water; however, we do not believe this has had an impact. We also did not test the use of a strip filled with distilled water in the top row, which also consisted of outer wells for which higher OD values were obtained than those in the other rows. Yet, as variation between the wells in the top row was low (mean CV of 1.1% in the second experiment) and the first and last well were not used, as they were filled with distilled water, this row can be used in index calculations as long as both samples are tested in wells in this row. Another limitation of our study was the inclusion of three CSF-serum pairs for which the criteria of the manufacturer for the calculation of the *Borrelia*-specific CSF/serum IgG quotient and the *Borrelia*-specific IgG AI were not fulfilled by using the adapted protocol. Ideally, these should have been repeated; however, as the measurements barely fell outside the manufacturers' criteria, we decided to use these results in the study. Moreover, we argue that these minor transgressions would probably be accepted and reported in routine clinical/laboratory practice. By simulating the standard protocol, the criteria for the calculation of the *Borrelia*-specific CSF/serum IgG quotient and the *Borrelia*-specific IgG AI were not met for 4 out of the 149 (for whom the CSF sample was simulated to be tested in an outer well) and for 9 out of 149 (for whom the serum was simulated to be tested in an outer well) CSF-serum pairs. In practice, by using the standard protocol, more dilutions would have been needed to obtain valid results for these cases.

The edge effect found in this study could be caused by differences in temperature across the plate, with lower temperatures in the center of the plate than in the edges, as has been described previously [5, 7, 15]. Increased temperatures at the edges will cause more evaporation in outer wells and results in higher OD values [16, 17]. Also, wells located at the edges are not fully surrounded by other wells, which can affect the temperature in those wells [15], and temperature differences between filled wells (higher) and empty wells (lower) have also been described [5]. Variations across the plate could also be caused by differences in surface properties or unequal protein binding [8, 15, 18].

We demonstrated that the observed edge effect found in the Enzygnost IgG ELISA could have an impact on LNB diagnostics, especially when CSF is tested in an outer well and serum in an inner well. The magnitude of this impact was shown by calculating simulated Enzygnost IgG ELISA results by using the results of 149 CSF-serum pairs that were part of a larger validation study (T. van Gorkom, W. Voet, G. H. J. van Arkel, M. Heron, S. F. T. Thijsen, and K. Kremer, unpublished data). Depending on which sample (either CSF or serum) was placed in an outer well, either 15 (10.1%) of the 149 CSF-serum pairs resulted in a false-pathological AI, or 2 (1.3%) resulted in a false-normal AI. The number of false-pathological and false-normal results obtained through simulation was based on a worst-case scenario. That is, the edge-effect factor that was used to calculate the simulated CSF or serum results was based on selecting the two columns for which the difference between the median OD values was largest. Subsequently, these simulated CSF or serum results were used to calculate the simulated *Borrelia*-specific CSF/serum IgG quotients and, hence, the simulated *Borrelia*-specific IgG AIs. All CSF results, or all serum results, were subjugated to this simulated worst-case edge effect. Both the false-pathological AI values and the false-normal AI values were located close to the AI cutoff value of 1.5. This value, which is established by the manufacturer, is most likely based on the use of wells that perform similarly. Yet our study demonstrates that the AI cutoff value is too low for those cases in which the CSF is subjected to the edge effect (i.e., tested in an outer well in the standard protocol) and the serum is not (i.e., tested in an inner well in the standard protocol). Likewise, the AI cutoff value is too high for those cases in which the serum is subjected to the edge effect (i.e., tested in an outer

well in the standard protocol) and the CSF is not (i.e., tested in an inner well in the standard protocol).

Our study suggests that by using the adapted protocol, false-pathological and false-normal AIs caused by the edge effect can be avoided. A study of Wang and Cheng, in which the concentration of the monoclonal antibody bevacizumab was measured in plasma of beagle dogs by using a similar platform (ELISA), also demonstrated an edge effect, and they showed that exclusion of the wells at the edges decreased this effect [9]. Our study underlines the importance of investigating the performance of a new ELISA before it is implemented in routine diagnostics, as is required, e.g., by the ISO 15189 accreditation [19], as plate variation may apply to other ELISAs as well and may also depend on the test system used.

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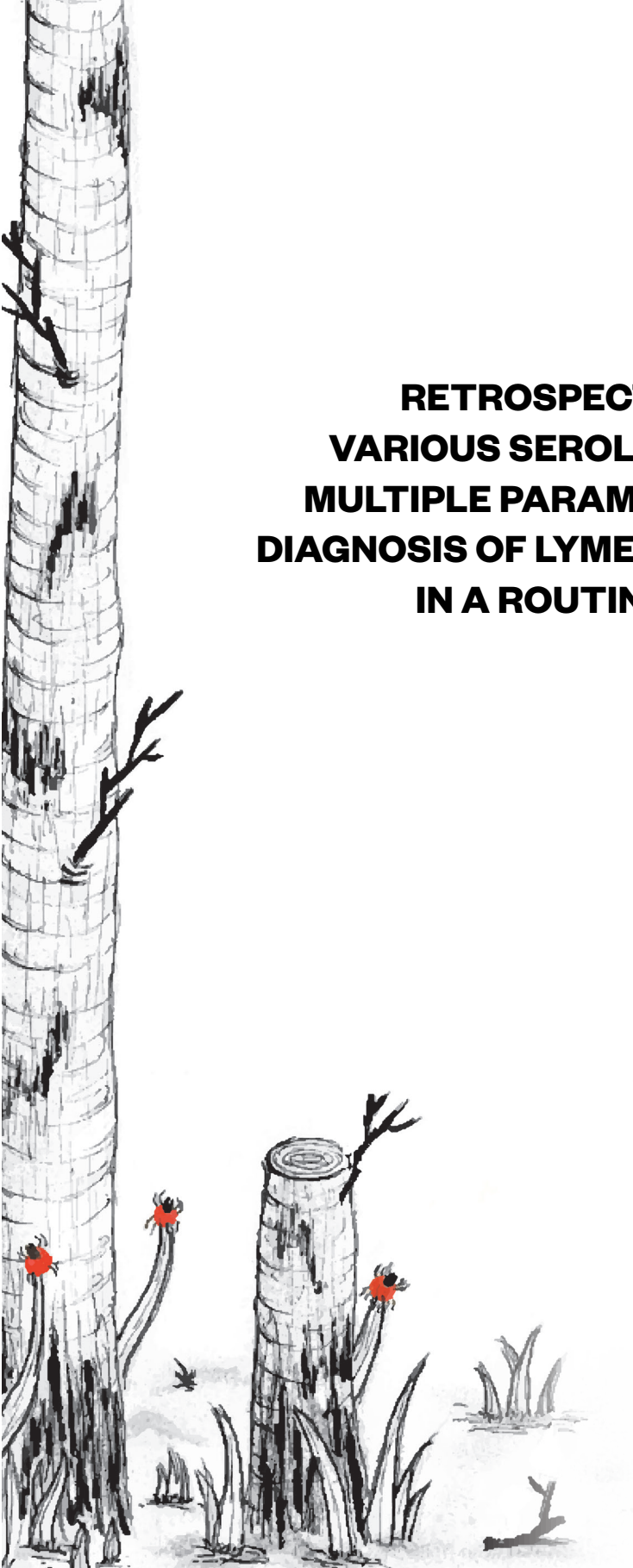

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RETROSPECTIVE EVALUATION OF VARIOUS SEROLOGICAL ASSAYS AND MULTIPLE PARAMETERS FOR OPTIMAL DIAGNOSIS OF LYME NEUROBORRELIOSIS IN A ROUTINE CLINICAL SETTING

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ABSTRACT

Laboratory diagnosis of Lyme neuroborreliosis (LNB) is challenging, and validated diagnostic algorithms are lacking. Therefore, this retrospective cross-sectional study aimed to compare the diagnostic performance of seven commercial antibody assays for LNB diagnosis. Random forest (RF) modeling was conducted to investigate whether the diagnostic performance using the antibody assays could be improved by including several routine cerebrospinal fluid (CSF) parameters (i.e., leukocyte count, total protein, blood-CSF barrier functionality, and intrathecal total-antibody synthesis), two-tier serology on serum, the CSF level of the B-cell chemokine (C-X-C motif) ligand 13 (CXCL13), and a *Borrelia* species PCR on CSF. In total, 156 patients were included who were classified as definite LNB (*n* = 10), possible LNB (*n* = 7), or non-LNB patient (*n* = 139) according to the criteria of the European Federation of Neurological Societies using a consensus strategy for intrathecal *Borrelia*-specific antibody synthesis. The seven antibody assays showed sensitivities ranging from 47.1% to 100% and specificities ranging from 95.7% to 100%. RF modeling demonstrated that the sensitivities of most antibody assays could be improved by including other parameters to the diagnostic repertoire for diagnosing LNB (range: 94.1% to 100%), although with slightly lower specificities (range: 92.8% to 96.4%). The most important parameters for LNB diagnosis are the detection of intrathecally produced *Borrelia*-specific antibodies, two-tier serology on serum, CSF-CXCL13, Reibergram classification, and pleocytosis. In conclusion, this study shows that LNB diagnosis is best supported using multiparameter analysis. Furthermore, a collaborative prospective study is proposed to investigate if a standardized diagnostic algorithm can be developed for improved LNB diagnosis.

IMPORTANCE

The diagnosis of LNB is established by clinical symptoms, pleocytosis, and proof of intrathecal synthesis of *Borrelia*-specific antibodies. Laboratory diagnosis of LNB is challenging, and validated diagnostic algorithms are lacking. Therefore, this retrospective cross-sectional study aimed to compare the diagnostic performance of seven commercial antibody assays for LNB diagnosis. Multiparameter analysis was conducted to investigate whether the diagnostic performance using the antibody assays could be improved by including several routine (CSF) parameters. The results of this study show that LNB diagnosis is best supported using the detection of intrathecally produced *Borrelia*-specific antibodies, two-tier serology on serum, CSF-CXCL13, Reibergram classification, and pleocytosis. Furthermore, we propose a collaborative prospective study to investigate the potential role of constructing a diagnostic algorithm using multiparameter analysis for improved LNB diagnosis.

KEYWORDS

Borrelia, Lyme neuroborreliosis, cerebrospinal fluid, intrathecal antibody synthesis, antibody index, two-tier serology, random forest, multiparameter analysis, Reibergram

Lyme borreliosis (LB), which is caused by *Borrelia burgdorferi* sensu lato, is the most common tick-borne disease in temperate regions of the Northern Hemisphere [1]. LB is a multisystem disease, and the most frequent clinical symptom is an expanding skin rash also known as erythema migrans [1, 2]. If untreated, the *Borrelia* bacterium can disseminate to other body parts, such as the peripheral and/or central nervous system (Lyme neuroborreliosis [LNB]), joints (Lyme arthritis), or heart (Lyme carditis), or cause acrodermatitis chronica atrophicans (ACA) [3]. In Europe and North America, LNB is seen in approximately 3% to 16% of LB cases [4-7] and often presents as a painful meningoradiculitis with or without cranial nerve involvement, although various combinations of other neurological complaints may occur as well [5, 8]. In the Netherlands, the annual incidence rate for LNB in 2010 was 2.6 per 100,000 inhabitants, which comprised one third of the total incidence rate of disseminated LB [9].

The diagnosis of LNB must be supported by laboratory tests, because the clinical symptoms of LNB are nonspecific. The European Federation of Neurological Societies (EFNS) recommends the detection of an intrathecal immune response to *B. burgdorferi* sensu lato together with the presence of pleocytosis (≥5 leukocytes/µl) in the cerebrospinal fluid (CSF) [5]. Proof of intrathecally produced *Borrelia*-specific antibodies requires simultaneous measurement of *Borrelia*-specific antibodies in CSF and serum of a CSF-serum pair, which should be interpreted relative to the total amount of antibodies in CSF and serum and taking the blood-CSF barrier functionality into consideration [5, 10]. The interpretation of the test results, however, can be complicated, as negative test results do not exclude LNB and positive test results are no indication of active disease. A negative test result in the first few weeks after infection can be explained by the absence of detectable antibody levels, which have to be built up at the start of the infection [11-13]. For antibody tests, sensitivities between 55% and 90% have been reported for symptom durations of less than 6 weeks [11, 14-18]. As the immune response against *Borrelia* expands over time [19-21], the sensitivity improves as the infection progresses and can ultimately reach 100% [11, 15, 22]. Lower sensitivities have also been reported for antibody assays that are based on a single antigen compared to those of antibody assays based on multiple antigens [18, 23, 24]. Furthermore, negative test results can be obtained when the antigens present in the assay do not match those of the *B. burgdorferi* sensu lato strain causing disease. This mismatch can be explained by the intra- and interspecies heterogeneity of *B. burgdorferi* sensu lato [25-30] and/or the antigenic variation the bacterium can apply during the course of disease [31]. A negative test result can also be caused by antibiotic treatment prior to the lumbar puncture (LP), as this might abrogate the immune response [32, 33]. A positive test result can be proof of an active LNB, but can also be the result of a previous, yet cleared, infection as antibody persistence has been reported after successful antibiotic treatment [34, 35].

In clinical practice, proof of intrathecal *Borrelia*-specific antibody synthesis for LNB diagnostics is based on either the detection of these antibodies in CSF-serum pairs and subsequent calculation of a *Borrelia*-specific antibody index (AI) [14-16, 18, 22, 24, 34, 36-38] or the detection of these antibodies in CSF only [39-41]. Many commercial assays are available for the detection of *Borrelia*-specific antibodies, and various studies have evaluated the performance of these assays for LNB diagnostics [16, 18, 23, 24, 38, 40]. A drawback of most of these studies is that study populations were used that were not representative of the clinical setting in which the antibody assays are used. Therefore, this study aimed to compare the diagnostic performance of seven commercial antibody assays for the diagnosis of LNB using a cross-sectional study design. Furthermore, a random forest (RF) model was constructed for each antibody assay to investigate whether the diagnostic performance found for each assay could be improved by including various routine CSF parameters (i.e., leukocyte count, total protein, blood-CSF barrier functionality, and intrathecal total-antibody synthesis). Other parameters added to each RF model included *Borrelia*-specific serum antibodies using two-tier serology, the CSF level of the B-cell chemokine (C-X-C motif) ligand 13 (CXCL13) [42], and a *Borrelia* species PCR on CSF.

RESULTS

STUDY POPULATION

In total, 150 (13.7%) of 1,098 consecutive patients who underwent at least one LP in the predefined study period were included in the current study. Six additional LNB patients were included from outside the predefined study period, all of whom had taken part in two other studies [43, 44]. Details on the selection of the 156 patients have already been published, these patients have also been used to evaluate two commercial CSF-CXCL13 assays [45].

CLASSIFICATION OF THE STUDY POPULATION USING THE EFNS GUIDELINES AND CONSENSUS STRATEGY

All patients were classified as definite LNB, possible LNB, or non-LNB patient based on the EFNS guidelines [5] and consensus strategy according to the flowchart in Fig. S1. Details with regard to clinical symptoms suggestive for LNB, an alternative diagnosis that ruled out LNB, and test results (i.e., pleocytosis and intrathecal *Borrelia*-specific antibody synthesis determined by the CSF-serum assays) needed for the classification of patients are shown in Table S2. The number of possible and definite LNB patients in this study differed slightly from that in our previous study [45], as intrathecal *Borrelia*-specific antibody synthesis was based on either a consensus strategy (this study) or the IDEIA results (previous study). Ten (6.4%) of the 156 patients were classified as definite LNB patient, and 7 (4.5%) of the 156 patients were classified as possible LNB patient (Table 1). Of the seven possible LNB patients, four (57.1%) had pleocytosis and three (42.9%) had intrathecal *Borrelia*-specific antibody synthesis according to the consensus strategy.

Table 1. Classification of the 156 study participants based on the guidelines of the European Federation of Neurological Societies (EFNS) [5] and consensus strategy.

EFNS criteria and consensus strategy used to classify the 156 study participants (no./total [%])				Classification of patients[a]		
Clinical symptoms suggestive of LNB[b]	**Pleocytosis (CSF leucocyte count ≥5 leucocytes/µl)**	**Consensus strategy for intrathecal *Borrelia*-specific Ab synthesis[c]**	**Other cause for symptoms**	**dLNB**	**pLNB**	**non-LNB**
Yes (56/156 [35.9])	Yes (17/56 [30.4])	Yes (10/17 [58.8])	0/10 (0.0)	10	0	0
		No (7/17 [41.2])	3/7 (42.9)[d]	0	4	3
	No (39/56 [69.6])	Yes (3/39 [7.7])	0/3 (0.0)	0	3	0
		No (36/39 [92.3])	-	0	0	36
No (100/156 [64.1])	Yes (19/100 [19.0])	Yes (0/19 [0.0])	-	0	0	0
		No (19/19 [100])	-	0	0	19
	No (81/100 [81.0])	Yes (0/81 [0.0])	-	0	0	0
		No (81/81 [100])	-	0	0	81
Total				10/156 (6.4)	7/156 (4.5)	139/156 (89.1)

a. Patients are classified as definite Lyme neuroborreliosis (dLNB), possible LNB (pLNB), or non-LNB patient based on the EFNS guidelines (3) and consensus strategy using the flow chart in Fig. S1.
b. Clinical symptoms suggestive of Lyme neuroborreliosis (LNB) were assumed to be present when a request for the detection of intrathecal *Borrelia*-specific antibody synthesis was done at our laboratory at the time of active disease in the past. Clinical symptoms suggestive for LNB or an alternative diagnosis that ruled out LNB as well as test results (i.e., pleocytosis and intrathecal *Borrelia*-specific antibody synthesis determined by the CSF-serum assays) needed for patient classification are shown in Table S2. For patients for whom clinical symptoms were not relevant for final classification, a diagnosis was specified in Table S2 only in case of a pathological immunoglobulin (Ig)M and/or IgG antibody index value in at least one of the five CSF-serum assays, and/or a positive test result in at least one of the two CSF-only assays, and/or a positive *Borrelia* species PCR result on CSF, and/or a positive CSF-CXCL13 result.
c. The consensus strategy entailed that intrathecal *Borrelia*-specific antibody (Ab) synthesis was considered proven only if the majority of the CSF-serum assays under investigation (i.e., IDEIA, Medac ELISA, *recom*Bead assay, Serion ELISA, and Enzygnost ELISA) showed a pathological *Borrelia*-specific IgM and/or IgG AI value (≥1.5).
d. For three patients, the diagnosis of LNB was ruled out, as another cause for their symptoms was found. One patient was diagnosed with neurosyphilis, one patient had residual complaints due to a previously treated LNB, and one patient had an isolated paralysis of the flexor pollicis due to a Schwannoma in the shoulder, see also Table S2.

A total of 139 (89.1%) of the 156 patients were classified as non-LNB patient. Thirty-nine (28.1%) of them had clinical symptoms suggestive of LNB, of whom 36 (92.3%) had neither pleocytosis nor intrathecal *Borrelia*-specific antibody synthesis according to the consensus strategy and 3 (7.7%) had pleocytosis only. For these three patients, another cause for their symptoms was found (Table S2). One hundred (71.9%) of the 139 patients classified as non-LNB patient did not have clinical symptoms suggestive of LNB. Eighty-one of them had neither pleocytosis nor intrathecal *Borrelia*-specific antibody synthesis according to the consensus strategy, and the remaining 19 had pleocytosis only.

DEMOGRAPHIC CHARACTERISTICS AND CLINICAL PARAMETERS AMONG THE THREE STUDY GROUPS

Table 2 shows a detailed overview of the demographic characteristics and clinical parameters among the three study groups. Pleocytosis, an elevated total protein level in CSF, and a positive *Borrelia* species PCR result on CSF was found among definite LNB patients more often than among possible LNB and non-LNB patients, and this was significantly more often than among non-LNB patients. CSF-CXCL13 positivity was found among definite LNB patients significantly more often than among possible LNB and non-LNB patients. No significant differences were observed among the three study groups with regard to gender, age, symptom duration, and CSF-glucose levels.

BORRELIA-SPECIFIC IGM AND IGG RESULTS ON SERUM AMONG THE THREE STUDY GROUPS

All definite and possible LNB patients had a positive (or equivocal) C6 enzyme-linked immunosorbent assay (ELISA) result on serum, and this was significantly higher than that among non-LNB patients (Table 2). Two-tier serology results on serum showed that the percentage of positive test results for *Borrelia*-specific immunoglobulin (Ig)M, IgG, and the combined IgM and IgG results, hereafter referred to as overall Ig results, was comparable between definite and possible LNB patients. These percentages were significantly higher among those two patient groups than among non-LNB patients, except for the percentage of positive test results for *Borrelia*-specific IgM between definite LNB and non-LNB patients.

BLOOD-CSF BARRIER FUNCTIONALITY AMONG THE THREE STUDY GROUPS

Measurement of CSF and serum albumin concentrations and the subsequent calculation of the CSF/serum quotient for albumin (Q Alb) provided insight into the functionality of the blood-CSF barrier. A dysfunctional blood-CSF barrier was found among definite LNB patients more often than among possible LNB and non-LNB patients, and this was significantly more often than among non-LNB patients (Table 2).

INTRATHECAL TOTAL IGM AND TOTAL IGG SYNTHESIS AMONG THE THREE STUDY GROUPS

Intrathecal total-antibody synthesis among definite LNB patients was based on an IgM with or without an IgG response (Table S2). Intrathecal total-antibody synthesis among possible LNB patients was based on a solitary IgM response and among non-LNB patients based on an IgM and/or IgG response. Intrathecal total IgM synthesis was found among definite and possible LNB patients significantly more often than among non-LNB patients (Table 2). Intrathecal synthesis of total IgG with or without total IgM was more common among definite LNB patients than among possible LNB and non-LNB patients, although this was significant only between definite LNB and non-LNB patients.

Table 2. Detailed overview of the demographic and clinical parameters among definite LNB, possible LNB, and non-LNB patients

	Value for indicated patient group[b]			Raw *P* value for BH comparison[b,e]		
Characteristic[a]	**dLNB (*n* = 10)[c]**	**pLNB (*n* = 7)[d]**	**non-LNB (*n* = 139)**	**dLNB vs pLNB**	**dLNB vs non-LNB**	**pLNB vs non-LNB**
Gender (no. of males [%])	7 (70.0)	5 (71.4)	66 (47.5)	1.000	0.203	0.266
Age (mean [95% CI]/[range])	61.2 (48.1-74.3)/(10.7-89.2)	54.1 (46.1-62.0)/(42.1-74.3)	51.8 (49.1-54.6)/(17.2-83.4)	0.133	0.063	0.740
Duration of symptoms in days (geometric mean [95% CI]/[range])[f]	26.3 (11.9-58.0)/(3.0-174)	51.9 (19.1-140)/(8.0-288)	64.1 (44.3-92.8)/(0.0-2,911)	0.364	0.075	0.633
Pleocytosis						
CSF leucocyte count ≥5 leucocytes/µl (no. [%])	10 (100)	4 (57.1)	22 (15.8)	0.051	<0.001[j]	0.019[k]
CSF leucocyte count/µl (geometric mean [95% CI]/[range])	76.7 (38.9-151)/(8.3-394)	6.7 (3.2-14.2)/(2.0-21.0)	1.1 (0.8-1.6)/(0.0-821)	<0.001[j]	<0.001[j]	0.001[j]
Glucose in CSF in mmol/l (geometric mean [95% CI]/[range])	3.3 (2.9-3.7)/(2.3-5.1)	3.8 (3.6-4.1)/(3.5-4.6)	3.6 (3.5-3.7)/(1.0-7.7)	0.012[k]	0.029[k]	0.127
Total protein in CSF						
Elevated total protein in CSF (yes [%])[g]	6 (60.0)	0 (0.0)	10 (7.2)	0.035[k]	<0.001[j]	1.000
Total protein in g/l (mean [95% CI]/[range])	940 (687-1,190)/(430-1,490)	461 (370-553)/(300-650)	450(389-512)/(170-4,280)	0.006[j]	<0.001[j]	0.279
Positive CXCL13 result on CSF	9 (90.0)	1 (14.3)	2 (1.4)	0.004[j]	<0.001[j]	0.138
Borrelia species PCR result on CSF	2 (20.0)	0 (0.0)	0 (0.0)	0.485	0.004[j]	1.000
C6 ELISA on serum	10 (100)	7 (100)	38 (27.3)	1.000	<0.001[j]	<0.001[j]
Two-tier serology on serum[h]						
Borrelia-specific IgM (no. [%])	3 (30.0)	3 (42.9)	5 (3.6)	0.644	0.010[k]	0.003[j]
Borrelia-specific IgG (rev)[h] (no. [%])	9 (90.0)	6 (85.7)	29 (20.9)[h]	1.000	<0.001[j]	<0.001[j]
Borrelia-specific IgG (old)[h] (no. [%])			27 (19.4)	1.000	<0.001[j]	<0.001[j]
Borrelia-specific IgM and/or IgG (rev)[h] (no. [%])	9 (90.0)	6 (85.7)	31 (22.3)[h]	1.000	<0.001[j]	0.001[j]
Borrelia-specific IgM and/or IgG (old)[h] (no. [%])			29 (20.9)	1.000	<0.001[j]	<0.001[j]
Albumin						
Dysfunctional blood-CSF barrier (no. [%])	9 (90.0)	3 (42.9)	22 (15.8)	0.101	<0.001[j]	0.097
Q albumin (mean x 10^{-3}) [95% CI]/[range])	13.6 (9.9-17.2)/(5.7-24.2)	7.0 (4.7-9.3)/(3.7-12.5)	6.1 (5.1-7.2)/(1.1-72.3)	0.019[k]	<0.001[j]	0.188
Intrathecal total antibody synthesis[i]						
Intrathecal total IgM (no. [%])	7 (70.0)	3 (42.9)	7 (5.0)	0.350	<0.001[j]	0.007[j]
Intrathecal total IgG (no. [%])	5 (50.0)	0 (0.0)	9 (6.5)	0.044[k]	<0.001[j]	1.000
Intrathecal total IgM and/or IgG (no. [%])	7 (70.0)	3 (42.9)	14 (10.1)	0.350	<0.001[j]	0.035[k]

a. CI, confidence interval; CSF, cerebrospinal fluid; CXCL13, B-cell chemokine (C-X-C motif) ligand 13; Q, quotient.
b. Patients are categorized as definite Lyme neuroborreliosis (dLNB), possible LNB (pLNB), or non-LNB patient based on the EFNS guidelines [5] and consensus strategy using the flow chart in Fig. S1.
c. Six (60.0%) of the 10 definite LNB patients were part of the consecutive patients included between August 2013 and June 2016, and 4/10 (40.0%) were selected from outside this period, see also Table S2.
d. Five (71.4%) of the seven possible LNB patients were part of the consecutive patients included between August 2013 and June 2016, and 2/7 (28.6%) were selected from outside this period, see also Table S2.
e. BH, Benjamini-Hochberg.
f. Durations of symptoms in days for definite and possible LNB patients are also listed in Table S2.
g. An elevated total protein concentration in the CSF is age dependent (reference range: 150 to 300 mg/ml for ages ≤10 years, 200 to 500 mg/ml for ages between 10 and 40 years, and 250 to 800 mg/ml for ages >40 years [73]).
h. Two-tier serology on serum was performed using the C6 ELISA as a screening test and positive (and equivocal) C6 ELISA results were confirmed using the *recom*Line IgM and IgG immunoblot (IB). The manufacturer of the *recom*Line IB revised the interpretation of the *recom*Line IgG IB in January 2019 by increasing the point value of the VlsE band (Table S3). For two non-LNB patients, the *recom*Line IgG IB result changed from negative to equivocal (equivocal results were scored positive), see also Table S2. Consequently, results are shown that include both the revised (rev) and old interpretation criteria.
i. Intrathecal total IgM and/or total IgG synthesis is proven if the intrathecal fraction is larger than 10% as described by Reiber (64).
j. Significant *P* value after applying the Benjamini-Hochberg procedure (FDR ≤ 2.0%).
k. Nonsignificant *P* value after applying the Benjamini-Hochberg procedure (FDR > 2.0%).

BORRELIA-SPECIFIC IGM AND IGG AI RESULTS AMONG THE THREE STUDY GROUPS

For each of the five CSF-serum assays, the percentage of positive AI results for *Borrelia*-specific IgM, IgG, or both among definite LNB patients was significantly higher than that among non-LNB patients (Table 3). Most of these percentages were also higher among possible LNB than among non-LNB patients, and this was significant for *Borrelia*-specific IgG and overall Ig, except when the IDEIA was used. For *Borrelia*-specific IgM, however, this was significant only using the Enzygnost IgM ELISA. For all definite LNB patients, and for all possible LNB patients without pleocytosis (n = 3), a positive *Borrelia*-specific AI result was based on an IgG response with or without IgM (Table S2). Of the possible LNB patients with pleocytosis (n = 4), two had a positive *Borrelia*-specific AI result for IgM only in either one or two CSF-serum assays. Twelve (8.6%) of the 139 non-LNB patients had a positive *Borrelia*-specific AI result for IgM and/or IgG in a minority of the CSF-serum assays (Table S2).

Table 3. Results of the five CSF-serum assays and two CSF-only assays among definite LNB, possible LNB, and non-LNB patients

		No. of cases with a positive result per total (%) for indicated patient group[a]			Raw *P* value for BH comparison[a,d]		
Assay	**Antibody class**	**dLNB (n = 10)[b]**	**pLNB (n = 7)[c]**	**non-LNB (n = 139)**	**dLNB vs pLNB**	**dLNB vs non-LNB**	**pLNB vs non-LNB**
IDEIA	IgM	2/10 (20.0)	1/7 (14.3)	0/139 (0.0)	1.000	0.004[i]	0.048[j]
	IgG	7/10 (70.0)	1/7 (14.3)	0/139 (0.0)	0.050	<0.001[i]	0.048[j]
	IgM and/or IgG	7/10 (70.0)	1/7 (14.3)	0/139 (0.0)	0.050	<0.001[i]	0.048[j]
Medac ELISA	IgM	4/10 (40.0)	1/7 (14.3)	0/139 (0.0)	0.338	<0.001[i]	0.048[j]
	IgG	10/10 (100)	3/7 (42.9)	0/139 (0.0)	0.015[j]	<0.001[i]	<0.001[i]
	IgM and/or IgG	10/10 (100)	4/7 (57.1)	0/139 (0.0)	0.051	<0.001[i]	<0.001[i]
*recom*Bead assay[e]	IgM	4/10 (40.0)	0/6 (0.0)[e]	0/139 (0.0)	0.234	<0.001[i]	1.000
	IgG	10/10 (100)	3/6 (50.0)[e]	4/138 (2.9)[e]	0.036[j]	<0.001[i]	0.001[i]
	IgM and/or IgG	10/10 (100)	3/5 (60.0)[e]	4/138 (2.9)[e]	0.095	<0.001[i]	<0.001[i]
Serion ELISA[f]	IgM	5/9 (55.6)[f]	1/7 (14.3)	0/138 (0.0)[f]	0.145	<0.001[i]	0.048[j]
	IgG	9/9 (100)[f]	3/7 (42.9)	6/138 (4.3)[f]	0.019[j]	<0.001[i]	0.005[i]
	IgM and/or IgG	9/9 (100)[f]	3/7 (42.9)	6/138 (4.3)[f]	0.019[j]	<0.001[i]	0.005[i]
Enzygnost ELISA[g]	IgM	3/5 (60.0)[g]	2/5 (40.0)[g]	1/139 (0.7)	1.000	<0.001[i]	0.003[i]
	IgG	5/5 (100)[g]	3/5 (60.0)[g]	3/139 (2.2)	0.444	<0.001[i]	<0.001[i]
	IgM and/or IgG	5/5 (100)[g]	5/5 (100)[g]	4/139 (2.9)	1.000	<0.001[i]	<0.001[i]
C6 ELISA	IgM and/or IgG	10/10 (100)	6/7 (85.7)	5/139 (3.6)	0.412	<0.001[i]	<0.001[i]
*recom*Line IB[h]	IgM	0/10 (0.0)	0/7(0.0)	0/139 (0.0)	1.000	1.000	1.000
	IgG (rev)[h]	8/10 (80.0)[h]	1/7 (14.3)[h]	5/139 (3.6)[h]	0.015[j]	<0.001[i]	0.259
	IgG (old)[h]	7/10 (70.0)	0/7 (0.0)	0/139 (0.0)	0.010[j]	<0.001[i]	1.000
	IgM and/or IgG (rev)[h]	8/10 (80.0)[h]	1/7 (14.3)[h]	5/139 (3.6)[h]	0.015[j]	<0.001[i]	0.259
	IgM and/or IgG (old)[h]	7/10 (70.0)	0/7 (0.0)	0/139 (0.0)	0.010[j]	<0.001[i]	1.000

a. Patients are classified as definite Lyme neuroborreliosis (dLNB), possible LNB (pLNB), or non-LNB patient based on the EFNS criteria [5] and consensus strategy using the flow chart in Fig. S1.
b. Six (60.0%) out of 10 dLNB patients were part of the consecutive patients included between August 2013 and June 2016, and 4/10 (40.0%) were selected from outside this period, see also Table S2.
c. Five (71.4%) out of seven pLNB patients were part of the consecutive patients included between August 2013 and June 2016, and 2/7 (28.6%) were selected from outside this period, see also Table S2.
d. BH, Benjamini-Hochberg.
e. For three cases, either the IgM AI value (one pLNB patient) or the IgG AI value (one pLNB and one non-LNB patient) could not be determined by the *recom*Bead assay due to insufficient material.
f. For two cases, one dLNB and one non-LNB patient, the IgM and IgG AI values could not be determined by the Serion ELISA due to insufficient sample material.
g. For seven cases, five dLNB and two pLNB patients, the IgM and IgG AI values could not be determined by the Enzygnost ELISA, because the ELISA was taken of the market.
h. The manufacturer of the *recom*Line immunoblot (IB) revised the interpretation of the *recom*Line IgG IB in January 2019 by increasing the point value of the VlsE band (Table S3). For seven cases, one dLNB, one pLNB, and five non-LNB patients, the *recom*Line IgG IB result changed from negative to equivocal (equivocal results were scored positive), see also Table S2. Consequently, results are shown that include both the revised (rev) and old interpretation criteria.
i. Significant *P* value after applying the Benjamini-Hochberg procedure (FDR ≤ 2.0%).
j. Nonsignificant *P* value after applying the Benjamini-Hochberg procedure (FDR > 2.0%).

BORRELIA-SPECIFIC IGM AND IGG IN CSF AMONG THE THREE STUDY GROUPS

All definite and most of the possible LNB patients had a positive C6 ELISA result on CSF, and this C6 ELISA positivity was significantly higher than that among non-LNB patients (Table 3). Using the *recom*Line immunoblot (IB) on CSF, IgM was not detected at all. *Recom*Line IgG IB positivity using the revised interpretation criteria of the manufacturer (Table S3), which were elaborated on throughout this article, was more common among definite LNB patients than among possible LNB and non-LNB patients, although this was significant only between definite LNB and non-LNB patients (Table 3).

COMPARISON OF *BORRELIA*-SPECIFIC IGM AND IGG IN CSF AND SERUM

Detection of *Borrelia*-specific antibodies in CSF is no direct proof that these antibodies are intrathecally produced and can also be explained by passive diffusion from the blood. C6 ELISA positivity in CSF among definite LNB patients did not differ from that in serum (100% [10/10], both) (Table S2). C6 ELISA positivity in CSF among possible LNB patients was comparable to that in serum (85.7% [6/7] and 100% [7/7], respectively) (P = 1.000). Among non-LNB patients, however, C6 ELISA positivity in CSF was significantly lower than that in serum (3.6% [5/139] and 27.3% [38/139], respectively) (P < 0.001; false-discovery rate [FDR] ≤ 2.0%).

The overall Ig *recom*Line IB positivity in CSF among definite LNB patients was comparable to that in serum (80.0% [8/10] and 90% [9/10], respectively) (Table S2) (P = 1.000). Among possible LNB patients, the overall Ig *recom*Line IB positivity in CSF was lower than that in serum (14.3% [1/7] and 85.7% [6/7], respectively) (P = 0.074). Among non-LNB patients, the overall Ig *recom*Line IB positivity in CSF was significantly lower than that in serum (3.6% [5/139] and 25.2% [35/139], respectively) (P < 0.001; FDR ≤ 2.0%).

DIAGNOSTIC PERFORMANCE OF THE SEVEN ANTIBODY ASSAYS

The sensitivities of the IgM assays to diagnose LNB among definite and possible LNB patients ranged from 0.0% (*recom*Line IgM IB) to 50.0% (Enzygnost IgM ELISA) (Fig. 1A). The sensitivities of the IgG assays were higher and ranged from 47.1% (IDEIA IgG) to 81.3% (*recom*Bead IgG assay) (Fig. 1A). The overall Ig sensitivities of the antibody assays largely overlapped with those for IgG only and ranged from 47.1% (IDEIA) to 100% (Enzygnost ELISA) (Fig. 1A). The overall Ig sensitivities of two assays were slightly higher than those for IgG only, as a solitary IgM response was found for two possible LNB patients in either one (Medac ELISA) or two (Medac and Enzygnost ELISA) assays (Table S2). The sensitivity of the *recom*Bead assay based on the overall Ig results was also slightly higher than that for IgG only, as for one possible LNB patient the IgM result was missing (Table S2). The specificities of all IgM assays were 100%, except for the Enzygnost IgM ELISA for which the specificity was 99.3% (Fig. 1A). The specificities of the IgG assays ranged from 95.7% (Serion IgG) to 100% (IDEIA IgG and Medac IgG) (Fig. 1A). The specificity of the Enzygnost ELISA based on the overall Ig results was slightly lower than that for IgG only due to a solitary IgM response for one non-LNB patient (Table S2). The sensitivity and specificity of the C6 ELISA were 94.1% and 96.4%, respectively. For IgM, IgG, and overall Ig, the sensitivity and specificity of the seven antibody assays did not differ significantly as the respective 95% confidence intervals (CIs) overlapped (Fig. 1A).

The positive predictive value (PPV) of the Enzygnost IgM ELISA was 83.3%, and the PPVs of all other IgM assays were 100%, except for the *recom*Line IgM IB, for which the PPV could not be calculated due to the absence of positive IgM results (Fig. 1B). For the IgG assays, the PPVs ranged from 64.3% (*recom*Line IgG IB) to 100% (IDEIA and Medac ELISA) (Fig. 1B). The PPVs of the antibody assays based on the overall Ig results were almost comparable to those based on IgG only (Fig. 1B). The negative predictive values (NPVs) of the IgM assays ranged from 89.1% (*recom*Line IgM IB) to 96.5% (Enzygnost IgM ELISA) (Fig. 1B). For the IgG assays, the NPVs ranged

from 93.9% (IDEIA IgG) to 98.6% (Enzygnost IgG ELISA) (Fig. 1B). The NPVs of the antibody assays based on the overall Ig results were almost comparable to those based on IgG only (Fig. 1B). The PPV and NPV of the C6 ELISA were 76.2% and 99.3%, respectively. For IgM, IgG, and the overall Ig results, the PPVs and negative predictive values (NPVs) of the seven antibody assays did not differ significantly as the respective 95% CIs overlapped (Fig. 1B).

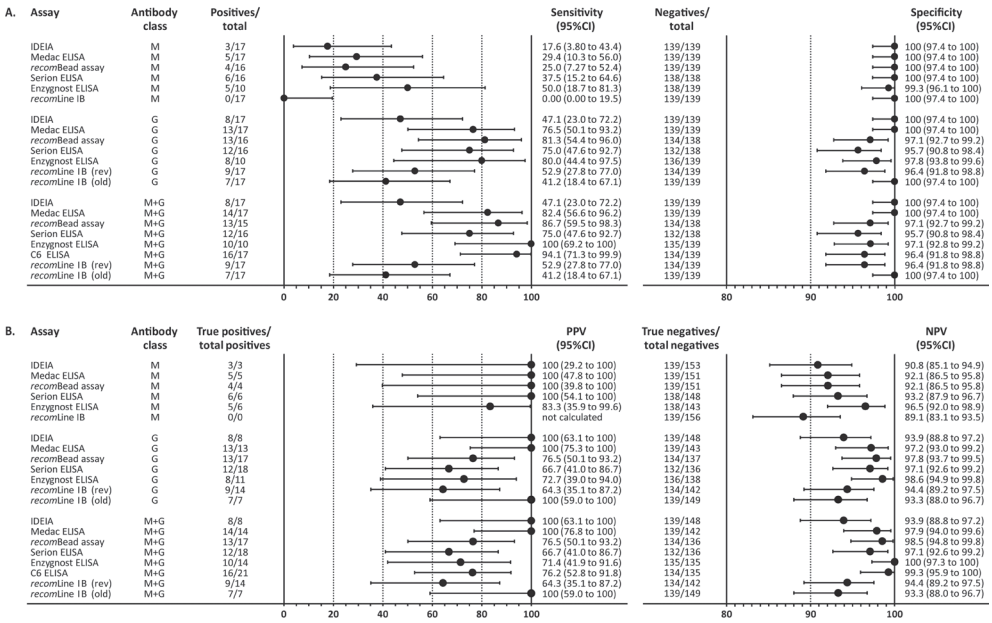

Fig. 1. Overview of the sensitivity and specificity (A) and the positive (PPV) and negative predictive value (NPV) (B) and 95% confidence intervals (CIs) of the five antibody assays tested on cerebrospinal fluid (CSF)-serum pairs and the two antibody assays tested on CSF only for IgM (M), IgG (G), or IgM and IgG combined (M+G). Cases consisted of definite and possible LNB patients, and controls consisted of non-LNB patients. The positives per total (A) are based on the number of pathological AI values (CSF-serum assays) or positive test results (CSF-only assays) among all the cases and are used to calculate the sensitivity. The negatives per total (A) are based on the number of normal AI values (CSF-serum assays) or negative test results (CSF-only assays) among all the controls and are used to calculate the specificity. The true positives (B) are cases that have either a pathological AI value (CSF-serum assays) or a positive test result (CSF-only assays) per total positives (i.e., all patients that have a pathological AI value [CSF-serum assays] or a positive test result [CSF-only assays]). The true negatives (B) are controls that have either a normal AI value (CSF-serum assays) or a negative test result (CSF-only assays) per total negatives (i.e., all patients that have a normal AI value [CSF-serum assays] or a negative test result [CSF-only assays]). The manufacturer of the *recom*Line immunoblot (IB) revised the interpretation of the *recom*Line IgG IB in January 2019 by increasing the point value of the VlsE band (Table S3), which had an effect on the test result (Table 3 and S2). Consequently, results are shown that include both the revised (rev) and old interpretation criteria. For the *recom*Line IgM IB, the PPV could not be calculated as this assay yielded no positive test results.

POTENTIAL ROLE OF ADDITIONAL PARAMETERS FOR PREDICTING LNB

Random forest (RF) modeling was performed to investigate if the diagnostic performance using the antibody assays could be improved by including the results of other parameters. The seven RF models performed comparably well, which was reflected by the areas under the curve (AUCs) that ranged from 0.970 to 0.993 (Table 4). The probability of misclassification of the 156 patients ranged from 3.4% (Enzygnost ELISA RF model) to 7.1% (IDEIA and *recom*Line IB RF models). The sensitivities and NPVs of most RF models were higher than the upper limit of the respective 95% CIs obtained using the results of the antibody assays only, except for the C6 and the Enzygnost ELISA (Table 4 and Fig. 1A and B). In contrast, the specificities and PPVs of most RF models were comparable with those of the antibody assays only, except for the IDEIA and the Medac ELISA, for

which the specificities and PPVs obtained using RF modeling were below the lower limit of the respective 95% CIs obtained using the results of the antibody assays only.

For each RF model, the relative importance of the 13 predictor variables was visualized in a heat map (Table 5). Overall, the predictor variables in each RF model ranked comparably. The most important diagnostic parameter in predicting LNB was the antibody assay with a mean rank of 1.7, followed by two-tier serology on serum (mean rank of 2.4) and CSF-CXCL13 (mean rank of 2.6). Of all Reibergram-based predictor variables, a dysfunctional blood-CSF barrier with intrathecal total-antibody synthesis (i.e., Reibergram area 3) was most important with a mean rank of 4.1 and preceded pleocytosis (mean rank of 5.1). A dysfunctional blood-CSF barrier in the absence of intrathecal total-antibody synthesis (i.e., Reibergram area 2) and the *Borrelia* species PCR on CSF contributed the least (Table 5).

Table 4. The performance characteristics obtained by constructing random forest models for each antibody assay to predict Lyme neuroborreliosis[a]

	Value of performance characteristics of antibody-assay specific RF models[b]					
Antibody assay	**AUC**	**pmc**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
IDEIA	0.973	7.1	94.1	92.8	61.5	99.2
Medac ELISA	0.991	5.1	100	94.2	68.0	100
*recom*Bead assay	0.993	4.6	100	94.9	68.2	100
Serion ELISA	0.986	5.2	100	94.2	66.7	100
Enzygnost ELISA	0.986	3.4	100	96.4	66.7	100
C6 ELISA	0.987	4.5	94.1	95.7	72.7	99.3
*recom*Line IB (rev)[b]	0.970	7.1	94.1	92.8	61.5	99.2
*recom*Line IB (old)[b]	0.972	7.1	94.1	92.8	61.5	99.2

a AUC, area under the curve; pmc, probability of misclassification; PPV, positive predictive value; NPV, negative predictive value; IB, immunoblot.
b Each random forest (RF) model included the following 13 predictor variables: the respective antibody assay, two-tier serology on serum, pleocytosis, CSF-CXCL13, total protein in CSF, *Borrelia* species PCR on CSF, and the seven predictor variables based on one or multiple areas of the Reibergram as shown in Table 6.
c The manufacturer of the *recom*Line immunoblot (IB) revised the interpretation of the *recom*Line IgG IB in January 2019 by increasing the point value of the VlsE band (Table S3), which had an effect on the test result (Table 3 and S2) Consequently, results are shown that include both the revised (rev) and old interpretation criteria.

Table 5. Heat maps of the relative contribution of the 13 predictor variables included in the random forest models to investigate their contribution in predicting Lyme neuroborreliosis

	Relative importance of each predictor variable for each (antibody-assay specific) RF model[b]								
Predictor variables included in the RF model[a]	**IDEIA**	**Medac ELISA**	***recom*Bead assay**	**Serion ELISA**	**Enzygnost ELISA**	**C6 ELISA**	***recom*Line (rev)[c]**	***recom*Line (old)[c]**	**Mean rank of predictor variable[d]**
Antibody assay	211	354	303	250	250	318	94	174	1.7
Two-tier serology on serum[e]	204	173	157	178	111	145	231	217	2.4
CSF-CXCL13	174	141	170	191	88	153	197	179	2.6
Reibergram; area 3[f]	146	115	131	118	123	115	145	147	4.1
Pleocytosis	125	123	91	137	41	139	148	128	5.1
Reibergram; all areas separately[f]	95	74	126	101	85	108	99	99	5.6
Reibergram; areas 3 and 4[f]	81	60	82	64	68	78	89	86	7.4
Reibergram; area 1[f]	60	55	115	92	61	90	69	65	7.6
Reibergram; areas 2 and 3[f]	63	46	78	72	56	59	62	63	8.9
Reibergram; area 4[f]	58	40	63	52	39	55	65	64	10.1
Total protein in CSF	43	40	47	18	-29	28	71	43	11.0
Reibergram; area 2[f]	28	10	43	37	32	18	18	27	12.1
Borrelia PCR on CSF	27	28	28	33	0	27	27	27	12.3

a. RF, random forest; CSF, cerebrospinal fluid; CXCL13, B-cell chemokine (C-X-C motif) ligand 13.
b. The relative importance of each predictor variable was calculated as described by Liaw and Wiener [72].
c. The manufacturer of the *recom*Line immunoblot (IB) revised the interpretation of the *recom*Line IgG IB in January 2019 by increasing the point value of the VlsE band (Table S3), which had an effect on the test result (Table 3 and S2). Consequently, results are shown that include both the revised (rev) and old interpretation criteria.
d. For each RF model, the 13 predictor variables were ranked based on their relative contribution from 1 (highest contribution) to 13 (lowest contribution). The mean rank of each predictor variable was calculated using the individual ranks obtained in each of the seven RF models and did not include the RF model of the *recom*Line IgG IB results based on the old interpretation criteria.
e. Two-tier serology on serum was performed using the C6 ELISA as a screening test, and positive (and equivocal) C6 ELISA results were confirmed using the *recom*Line IgM and IgG IB. The two-tier serology results on serum included the *recom*Line IB results obtained with the revised interpretation criteria of the *recom*Line IgG IB (Table 2 and S2).
f. For each RF model, the contribution of the Reibergram classification was assessed as described in Table 6.

DISCUSSION

In this retrospective study, the diagnostic performance of seven antibody assays for the diagnosis of LNB was evaluated. A clinically well-defined study population was used consisting of all consecutive patients from whom CSF and serum were drawn in the routine clinical setting of our hospital and who fulfilled the inclusion criteria. Patients were classified using the EFNS guidelines [5], and intrathecal *Borrelia*-specific antibody synthesis was considered proven using a consensus strategy. RF modeling was performed to assess the utility of additional parameters for predicting LNB.

Of all performance characteristics determined in this study (i.e., sensitivity, specificity, PPV, and NPV), the sensitivity of the seven antibody assays to diagnose LNB among definite and possible LNB patients showed the largest variation (range: 47.1% to 100%), although none of the differences were statistically significant. In general, differences in sensitivity between antibody

assays can be influenced by several factors, such as the antigens present in the assay [24, 38, 46]. These antigens might be expressed at different time points [20, 21] or might not match the antigens expressed by the *B. burgdorferi* sensu lato strain causing disease due to inter- and intraspecies heterogeneity and/or antigenic variation [25-31]. Overall, it is reasonable to assume that antibody assays based on multiple antigens or whole-cell lysates are expected to give rise to a higher number of positive test results among cases than assays based on a single or a limited number of antigens. Indeed, the sensitivity of the IDEIA, based on a single antigen, was the lowest (i.e., 47.1%) and the sensitivity of the Enzygnost ELISA, based on a whole-cell lysate, was the highest (i.e., 100%). Other studies that investigated multiple antibody assays based on one (i.e., the IDEIA) or multiple antigens also reported the lowest sensitivity for the IDEIA [18, 23, 24].

Besides the large variation in sensitivity between the antibody assays, the sensitivity of most assays did not reach 100%, and this could be explained by the case definition used. In this study, both definite and possible LNB patients were included as cases, which is preferable from a clinical point of view to avoid undertreatment of LNB patients. It was hypothesized previously that possible LNB patients with pleocytosis most likely represent early LNB patients for whom the expanding antibody response is below the detection limit of the antibody assay [47, 48]. In this study, this hypothesis is supported by the presence of a solitary *Borrelia*-specific IgM response in two possible LNB patients with pleocytosis, underlining the need for both IgM and IgG testing in LNB diagnostics, as was mentioned before [14, 21]. This hypothesis is further supported by a paper from Hansen and Lebech [15], who also reported a low sensitivity for the IDEIA among LNB patients with a short disease duration (sensitivity of 17% for symptoms duration of ≤7 days), which increased to 100% for LNB patients with a disease duration of more than 6 weeks. Early antibiotic treatment can also affect sensitivity since it can abrogate the immune response and, consequently, result in (false) negative test results among cases; however, in this study, antibiotic treatment for LNB had started after the LP was performed (Table S2).

When antibody assays are used that are based on multiple antigens or whole-cell lysates, more (false) positive test results can be expected among controls as well, which leads to a lower specificity. Positive test results among non-LNB patients, which were found mainly for the IgG CSF-serum assays, indeed underline the positive correlation between the number of antigens present in the assay and the number of positive test results. Furthermore, antibody assays based on whole-cell lysates can generate false-positive test results due to the presence of cross-reactive antigens [49]. Two non-LNB patients, one with active neurosyphilis and one who had been treated for active neurosyphilis in the past, had a positive *Borrelia*-specific AI result in either the Serion IgG or the Enzygnost IgG ELISA, which could be explained by cross-reactive *Treponema* antibodies [16, 50]. As none of the non-LNB patients with a positive *Borrelia*-specific AI result had pleocytosis, except for the patient with active neurosyphilis, an active LNB infection was not likely [47, 48].

Considering that the use of a CSF-serum assay and the calculation of an AI is rather complicated, an assay tested on CSF only would be more convenient and preferable in routine clinical practice. The interpretation of positive test results using a CSF-only assay, however, is complicated because a positive test result can be caused by intrathecal *Borrelia*-specific antibody synthesis or be the result of passive diffusion of these antibodies from the blood or of a traumatic LP. Of the two CSF-only assays tested in this study, the C6 ELISA performed best and might be useful as a screening assay since the NPV was 99.3%. Positive C6 ELISA results on CSF, however, should be confirmed using a CSF-serum assay and subsequent AI calculation that corrects for a dysfunctional blood-CSF barrier to prove intrathecal *Borrelia*-specific antibody synthesis.

In addition to intrathecal *Borrelia*-specific antibody synthesis, the results of other parameters, such

as an elevated CSF leucocyte count [5, 14, 28], a dysfunctional blood-CSF barrier [5, 14], intrathecal total-antibody synthesis with an IgM dominance [14, 28, 51], and elevated CSF-total protein [5, 14, 28] and CSF-CXCL13 levels [5, 28, 42, 45], can support the diagnosis of LNB. In our study, these findings were confirmed, as all these parameter results were found among definite LNB patients more often than among non-LNB patients. These findings, thus, strengthen the correct classification of the patients in our study and prompted us to assess the additional value of these parameters in the diagnosis of LNB. RF modeling showed that additional parameters could, indeed, be helpful in the diagnosis of LNB by increasing the sensitivity and NPV, although with a loss in specificity and PPV. In clinical practice, however, overtreating some patients at the cost of not missing true LNB patients is preferred. Overall, two-tier serology on serum, CSF-CXCL13, a dysfunctional blood-CSF barrier with proof of intrathecal total-antibody synthesis (Reibergram area 3), and pleocytosis contributed the most to the increased diagnostic performance. To minimize undertreatment, antibody assays with a high NPV are preferred. The EFNS recommends using an AI calculation to prove intrathecal synthesis of *Borrelia*-specific antibodies [5], and the need for this is confirmed in our study. The NPVs of the antibody assays only and those of the RF models showed that RF modeling using a Reiber-based CSF-serum assay is preferred, as the respective NPVs were highest. The results obtained with RF modeling are promising and open up the possibility of defining a diagnostic algorithm for LNB diagnostics.

This study had some limitations. First, some CSF-serum assays lacked results for a few patients, which could have influenced the test performance of these assays. Second, due to the low LNB incidence, few LNB patients were included within the predefined study period. Therefore, six additional LNB patients were included from outside this period. As the total number of LNB patients included in the current study was comparable to the 15 patients expected to be diagnosed with LNB in the predefined study period, we believe that the cross-sectional design of the study holds as has been discussed in more detail previously [45]. Third, 20.9% of the non-LNB patients were seropositive for *Borrelia*-specific IgG, whereas the IgG seroprevalence of the Dutch population is 4% to 8% [52]. This suggests a selection bias in our study population, although one could argue that a neurologist is more inclined to perform an LP when *Borrelia*-specific antibodies are detected in the blood. This has no consequence for the evaluation of the seven antibody assays, since this reflects routine clinical practice and underlines the need of nonbiased, consecutively selected patient samples for the evaluation of diagnostic assays [53]. Fourth, a bias toward older patients was introduced in this study by the inclusion criteria, as at least 1,250 ml of CSF and 110 ml of serum had to be present before a patient could be included in order to perform the multiple antibody assays under investigation. In general, less patient material is collected from children than from adults. Indeed, of all the 423 consecutive patients from whom a CSF and serum sample was drawn less than 24 h apart (see Fig. 1 of the previously published manuscript [45]), 61 (14.4%) were children (age <18 years; data not shown). In contrast, of the 150 consecutive patients that had sufficient patient material and were included in this study (see Fig. 1 of the previously published manuscript [45]), only 2 (1.3%) were children (age <18 years; data not shown).

Between the start and publication of this study, some antibody assays that performed well in this study (i.e., Enzygnost, Medac, and C6 ELISA) have been taken off the market. This was partly caused by the new, more stringent quality requirements for in vitro diagnostics, which triggered manufacturers to discontinue the production of these assays because of increased costs [54]. This development might result in a movement toward the development of monopolies offering diagnostic assays, limiting the diagnostic repertoire [55] and making the availability of assays vulnerable, as was shown recently during the severe acute respiratory syndrome coronavirus 2 pandemic [56].

The main strengths of this study are the cross-sectional design [53] and the well-defined study population. The results obtained in this study should be confirmed, preferably using a prospective design, aiming at including more patients. Because of the relatively low LNB incidence, this is ideally done in an (inter)national joint collaboration using a multiparameter diagnostic algorithm in an effort to standardize LNB diagnostics. Furthermore, this study shows that the Serion ELISA is a suitable assay for the detection of intrathecal *Borrelia*-specific antibody synthesis, which, to our knowledge, has not been reported before.

In conclusion, this study shows that LNB diagnostics is best supported using an approach that includes the detection of intrathecally produced *Borrelia*-specific antibodies using a Reiber-based AI calculation, two-tier serology on serum, CSF-CXCL13, Reibergram classification, and pleocytosis. Furthermore, a collaborative prospective study is proposed to investigate if a standardized diagnostic algorithm can be developed using multiparameter analysis for improved LNB diagnosis.

MATERIALS AND METHODS

STUDY POPULATION

Retrospectively, and regardless of their clinical presentation and age, all consecutive patients were eligible for inclusion if a CSF sample and a blood sample (drawn within 24 h of the LP) had been sent to the microbiology laboratory of the Diakonessenhuis Hospital, Utrecht, the Netherlands in the period between August 2013 and June 2016. Until the start of this study in 2017, leftover material from these patients had been stored at -20°C and/or -80°C. Prior to the start of this study, all samples had been freeze-thawed once to aliquot for use in the various assays in this study as well as for another study [45], which ran in parallel. Aliquoted samples were stored at -20°C until use. A prerequisite for patients to be included was the availability of at least 1,250 ml of CSF and 110 ml of serum. Patients from whom the CSF was (visually) hemolytic or who had received treatment with intravenous IgG were excluded from the study, as both could interfere with the test results [57, 58]. One patient was excluded because of unreliable test results that implied a sample mix-up. As the final number of LNB patients among the included consecutive patients was limited, we included six patients from outside the predefined study period (from February 2011 to July 2013 and from July 2016 to November 2017) that had been diagnosed with LNB in our hospital. These additional LNB patients had taken part in two other studies of our research group for which only adult patients had been included [43, 44]. Due to these previous studies, both CSF and serum from the time of diagnosis had been stored. For the CSF and serum of these additional LNB patients, the same inclusion criteria applied as for the CSF and serum of all consecutive patients.

All CSF-serum pairs used in this study were anonymized. Approval of the local ethics committee was not necessary, as the main goal of our study was to compare various antibody assays and assess if additional parameters could improve LNB diagnostics for which leftover material can be used. We did, however, obtain approval from the hospital board. The results of this study are reported following the guidelines for diagnostic accuracy studies [59].

CLASSIFICATION OF THE STUDY POPULATION

The EFNS guidelines [5] were used to classify the patients in the current study. Following these guidelines, definite LNB patients should have (i) clinical symptoms suggestive of LNB in the absence of another cause, (ii) CSF pleocytosis (≥5 leukocytes/µl), and (iii) intrathecal synthesis of *Borrelia*-specific antibodies. Possible LNB patients should have clinical symptoms suggestive of LNB with either pleocytosis or intrathecally produced *Borrelia*-specific antibodies. Clinical symptoms

suggestive of LNB (i.e., fulfillment of the first EFNS criterion) were assumed to be present when a request for the detection of intrathecal *Borrelia*-specific antibody synthesis was done at our laboratory at the time of active disease in the past for which the IDEIA LNB IgM and IgG assay (Oxoid, Hampshire, United Kingdom) was used. If the second and/or third criterion (i.e., pleocytosis and intrathecal *Borrelia*-specific antibody synthesis, respectively) was also fulfilled, another cause for the symptoms had to be excluded according to the EFNS guidelines. To minimalize the bias in patient classification by using only the IDEIA results, a consensus strategy for proof of intrathecal synthesis of *Borrelia*-specific antibodies was applied. This strategy entailed that intrathecal *Borrelia*-specific antibody synthesis was considered proven only if the majority of the antibody assays tested on CSF-serum pairs in this study showed a pathological *Borrelia*-specific (IgM and/or IgG) AI value (Fig. S1).

BORRELIA-SPECIFIC ANTIBODY DETECTION IN CSF-SERUM PAIRS AND CSF ONLY

Seven commercial antibody assays were selected, which were based on different techniques, different *Borrelia* antigens, and different quantification methods (Table S3). For five assays, referred to here as CSF-serum assays, the detection of intrathecally produced *Borrelia*-specific IgM and/or IgG was done by testing the CSF and serum of each patient simultaneously. Results of these CSF-serum assays were used to calculate a *Borrelia*-specific AI value as described below. For two assays, *Borrelia*-specific IgM and/or IgG antibodies were determined in CSF only and are referred to here as CSF-only assays. The seven antibody assays were performed according to the respective manufacturer's instructions, unless otherwise specified here.

The first CSF-serum assay was the second-generation IDEIA LNB assay (Oxoid), referred to here as IDEIA, for the detection of IgM and IgG (Table S3) [44]. As the IDEIA was part of the routine LNB diagnostics in our hospital, CSF-serum pairs of patients for whom LNB was suspected had already been tested with this assay at the time of active disease in the past. Consequently, these results were retrieved from the laboratory information system. The CSF-serum pairs of patients for which a request for the detection of intrathecal *Borrelia*-specific antibody synthesis was not done at the time of active disease in the past were tested in batch. CSF-serum pairs were tested only in the singular due to the limited amount of sample material, except CSF-serum pairs for which the CSF sample had an optical density (OD) value of ≥0.100, which were repeated. The second CSF-serum assay was the *Borrelia* ELISA Medac (Medac GmbH, Hamburg, Germany), referred to here as Medac ELISA, for the detection of IgM and IgG (Table S3). The third CSF-serum assay was the *recom*Bead *Borrelia* 2.0 multiplex bead-assay (Mikrogen Diagnostik GmbH, Neuried, Germany), referred to here as the *recom*Bead assay, for the detection of IgM and IgG (Table S3). For both the Medac ELISA and the *recom*Bead assay, CSF-serum pairs with an equivocal AI value ($1.3 \leq AI < 1.5$) should have been repeated following the manufacturers' protocol (Table S3), but this was not done due to limited sample material. The fourth CSF-serum assay tested was the *Borrelia burgdorferi* SERION ELISA classic (Institute Virion/Serion GmbH, Würzburg, Germany), referred to here as Serion ELISA, for the detection of IgM and IgG (Table S3). The fifth CSF-serum assay was the Enzygnost Borreliosis/IgM ELISA (Siemens Healthcare Diagnostics, Marburg, Germany) and the Enzygnost Lyme link VlsE/IgG ELISA (Siemens Healthcare Diagnostics), referred to here as the Enzygnost ELISA (Table S3). CSF-serum pairs were tested using an adapted protocol we described previously [60], as an edge effect was established for the Enzygnost IgG ELISA following the standard protocol of the manufacturer. This adapted protocol was also used for the Enzygnost IgM ELISA, for which an edge effect was established as well (data not published).

The first CSF-only assay was the C6 ELISA (Immunetics, Boston, MA, USA), which measures total Ig (IgM and IgG) (Table S3) [61]. The protocol of the manufacturer describes its use only for serum. This protocol was used to test the CSF using a 1:5 dilution, similar to the CSF dilution used by van Burgel et al. [40]. The second CSF-only assay was the *recom*Line IB (Mikrogen GmbH) for the

detection of IgM and IgG (Table S3). For practical reasons, we used the manufacturer's protocol for serum to test the CSF. For testing CSF, however, we used a 1:10 dilution for IgM and a 1:20 dilution for IgG (for serum, a 1:51 dilution for IgM and a 1:101 dilution for IgG is recommended). In January 2019, the manufacturer of the *recom*Line IB revised the interpretation of the *recom*Line IgG IB (Table S3). Both the old and revised interpretation criteria were investigated in the current study [62, 63]; however, only the revised interpretation criteria were elaborated on throughout the article. For both the C6 ELISA and the *recom*Line IB, equivocal results were interpreted as positive.

All ELISAs were performed on a Dynex DS2 automated ELISA instrument (Dynex Technologies, Chantilly, VA, USA) and analyzed with the DS-Matrix software (Dynex Technologies). The *recom*Bead assay was performed on a Bio-Plex 200 instrument using the Luminex xMAP technology (Bio-Rad Laboratories, Hercules, CA, USA) and the Bio-Plex Manager software version 6.1 (Bio-Rad Laboratories). The *recom*Line IB was performed on an Autoblot 3000 (Medtec Biolab Equipment, Hillsborough, NC, USA). Subsequently, *recom*Line IB strips were scanned and the intensity of the bands was recorded using *recom*Scan software version 3.4 (Mikrogen GmbH).

BLOOD-CSF BARRIER FUNCTIONALITY AND INTRATHECAL TOTAL-ANTIBODY SYNTHESIS

To investigate the blood-CSF barrier functionality and the intrathecal total IgM and total IgG synthesis, CSF and serum concentrations of albumin, total IgM, and total IgG were determined at the start of this study by nephelometry and used to calculate the CSF/serum quotients for Q Alb, total IgM (Q IgM), and total IgG (Q IgG) as described previously [45]. The Q Alb is used to assess the functionality of the blood-CSF barrier as albumin originates from the blood, and a dysfunctional blood-CSF barrier is proven if the Q Alb exceeds the age-dependent Q Alb [10]. The Q IgM and Q IgG are used to assess intrathecal total-antibody synthesis. If either one or both quotients show a larger increase than the Q Alb and the intrathecal fraction of total IgM and/or total IgG is larger than 10%, then intrathecal total-antibody synthesis is proven [64].

CALCULATION OF THE *BORRELIA*-SPECIFIC AI

For the CSF-serum pairs, either one of two calculation methods was used to calculate the *Borrelia*-specific IgM and IgG AI value (Table S3). For the IDEIA, intrathecal *Borrelia*-specific antibody synthesis was proven if the fraction of *Borrelia*-specific IgM (or IgG) in the CSF as part of the total amount of IgM (or IgG) in the CSF exceeded that of serum, which is expressed by a *Borrelia*-specific IgM (or IgG) AI value (Table S3). Following the manufacturer's protocol, a *Borrelia*-specific AI value of ≥0.3 was considered pathological, and a *Borrelia*-specific AI value of <0.3 (or an OD value of CSF of <0.150) was considered normal. Because the IDEIA is based on a capture ELISA, a correction for a dysfunctional blood-CSF barrier was not necessary [15]. For the other four CSF-serum assays, intrathecal *Borrelia*-specific antibody synthesis was proven by the calculation of a *Borrelia*-specific IgM and IgG AI value according to Reiber and Peter [10]. These *Borrelia*-specific AI values were calculated by dividing the CSF/serum quotient of *Borrelia*-specific IgM (or IgG) by Q IgM (or Q IgG) by considering the blood-CSF barrier functionality. As the interpretation criteria shown in Table S3 differed slightly between the four CSF-serum assays, the cutoff for intrathecal pathogen-specific (IgM and/or IgG) antibody synthesis as described by Reiber was used [65]. Thus, *Borrelia*-specific AI values of ≥1.5 are considered pathological, *Borrelia*-specific AI values between 0.6 and 1.3 are considered normal, and *Borrelia*-specific AI values of <0.5 are not valid. In the current study, *Borrelia*-specific AI values between 1.3 and 1.5 were considered normal. Furthermore, *Borrelia*-specific AI values were used only if these were above the assay-specific lower cutoff (Table S3).

BORRELIA-SPECIFIC ANTIBODY DETECTION IN SERUM

Borrelia-specific antibodies in serum were determined previously using a two-tier protocol in which the C6 ELISA was used as a screening test and equivocal and positive C6 ELISA results were confirmed using the *recom*Line IB [45]. The *recom*Line IB was also performed on C6 ELISA negative sera in order to compare the *recom*Line IB results obtained in CSF with those obtained in serum to gain insight into the origin of the *Borrelia*-specific antibodies. Similar to the *recom*Line IgG IB performed on CSF, for serum we also applied the old and revised interpretation criteria of the *recom*Line IgG IB (Table S3) [62, 63]; however, only the revised interpretation criteria were elaborated on throughout the article.

CLINICAL SYMPTOMS AND ADDITIONAL PARAMETERS

Results from a number of other parameters, obtained at the time of active disease in the past, were retrieved from the patient and/or laboratory information system. These results included information about clinical symptoms, total protein and glucose concentrations in the CSF, and CSF leukocyte counts. For CSF samples with elevated erythrocyte counts (i.e., ≥1,000 erythrocytes/µl), the CSF leukocyte count was corrected by subtracting 1 leukocyte/µl for each 1,000 erythrocytes/µl according to Reiber and Peter [10]. For patients classified as definite or possible LNB patient, information regarding symptom duration and antibiotic treatment for LNB was retrieved from the patient information system.

An in-house *Borrelia* species PCR and two CXCL13 assays on CSF had been performed previously, of which the respective methods and results have been published [45]. For the current study, the final CSF-CXCL13 result was based on the combined result of the two CXCL13 assays and was considered negative when either one or both assays were negative and positive when both assays were positive.

STATISTICAL ANALYSIS

For all assays that determined IgM and IgG separately, the overall Ig results were based on a combination of the results of both immunoglobulins: negative when both IgM and IgG were negative and positive when at least one of these was positive. For all statistical analyses, Rstudio (version 1.4.1717, 2009 to 2021) was used [66]. We analyzed all data by performing two-group comparisons. The Fisher's exact test for count data was used for unpaired nominal data, the McNemar's chi-squared test with continuity correction was used for paired nominal data, and the exact Wilcoxon-Mann-Whitney test was used for quantitative unpaired data in a 2 by 2 table using the "stats" package [67]. Unpaired nominal data in a 2 by 4 table were analyzed with the approximate Monte Carlo resampling 10^6 Pearson's chi-squared test using the "coin" package [68]. Depending on the distribution, either the (geometric) mean value with the 95% CI or the median value and range were shown. Raw *P* values of <0.050 were statistically significant; however, they were interpreted after correction for the multiple statistical analyses in this study, for which the Benjamini-Hochberg procedure (BH) was applied [69]. The false-discovery rate (FDR) was set at the level of 2.0% (i.e., less than one false-positive test result was allowed in our list of rejections).

For each antibody assay, the sensitivity, specificity, PPV, and NPV with 95% CIs were calculated using the "epiR" package [70], for which definite and possible LNB patients were used as cases and all non-LNB patients were used as controls.

To investigate if the diagnostic performance using the antibody assays could be improved by including the results of other parameters, RF modeling was performed to predict LNB [71]. For each antibody assay, an RF model was built using the "randomForest" package [72] which included 13 predictor variables. The first six predictor variables comprised (i) one of the seven

antibody assays (negative/positive), (ii) two-tier serology on serum (negative/positive), (iii) pleocytosis (no/yes), (iv) CSF-CXCL13 (negative/positive), (v) elevated total protein in CSF (no/yes), and (vi) *Borrelia* species PCR on CSF (negative/positive). Blood-CSF barrier functionality and intrathecal total-antibody synthesis were also included in the RF models for which the previously published Reibergram classification was used [45]. In short, all patients included in this study were classified to one of four of the five Reibergram areas: Reibergram area 1, a normal blood-CSF barrier without intrathecal total IgM and/or IgG synthesis of >10% (*n* = 107/156), Reibergram area 2, a dysfunctional blood-CSF barrier without intrathecal total IgM and/or IgG synthesis of >10% (*n* = 25/156), Reibergram area 3, a dysfunctional blood-CSF barrier with intrathecal total IgM and/or IgG synthesis of >10% (*n* = 9/156), and Reibergram area 4, intrathecal total IgM and/or IgG synthesis of >10% with a normal blood-CSF barrier (*n* = 15/156) (Table S2). Subsequently, seven predictor variables were constructed based on one or multiple Reibergram areas (Table 6).

For all RF models, definite and possible LNB patients were defined as cases and all non-LNB patients were defined as controls. In total, 10^5 decision trees were built, and for each tree node, three predictor variables were used to split the tree. The predictions obtained in each RF model were used to construct a receiver operating characteristic (ROC) curve, which was subsequently used to calculate the AUC. For each RF model, the optimal cutoff for predicting LNB was calculated using the point on the ROC curve closest to the upper left corner, where both sensitivity and specificity are 100%, and this was determined by the square root of $[(1\text{-sensitivity})^2 + (1\text{-specificity})^2]$. Using the optimal cutoff value, the sensitivity, specificity, PPV, and NPV of each RF model were calculated. As the performance characteristics are based on predictions, 95% CIs were not calculated. For each RF model, the probability of misclassification and the relative importance of each predictor variable were calculated as described by Liaw and Wiener [72]. For each RF model, the relative importance of the 13 predictor variables was made visible by construction of a heat map. Subsequently, for each RF model, the predictor variables were ranked from 1 (highest relative contribution) to 13 (lowest relative contribution). These ranks were then used to calculate the mean rank of each predictor variable for the seven RF models to assess the importance of each predictor variable in predicting LNB.

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SUPPLEMENTAL MATERIAL

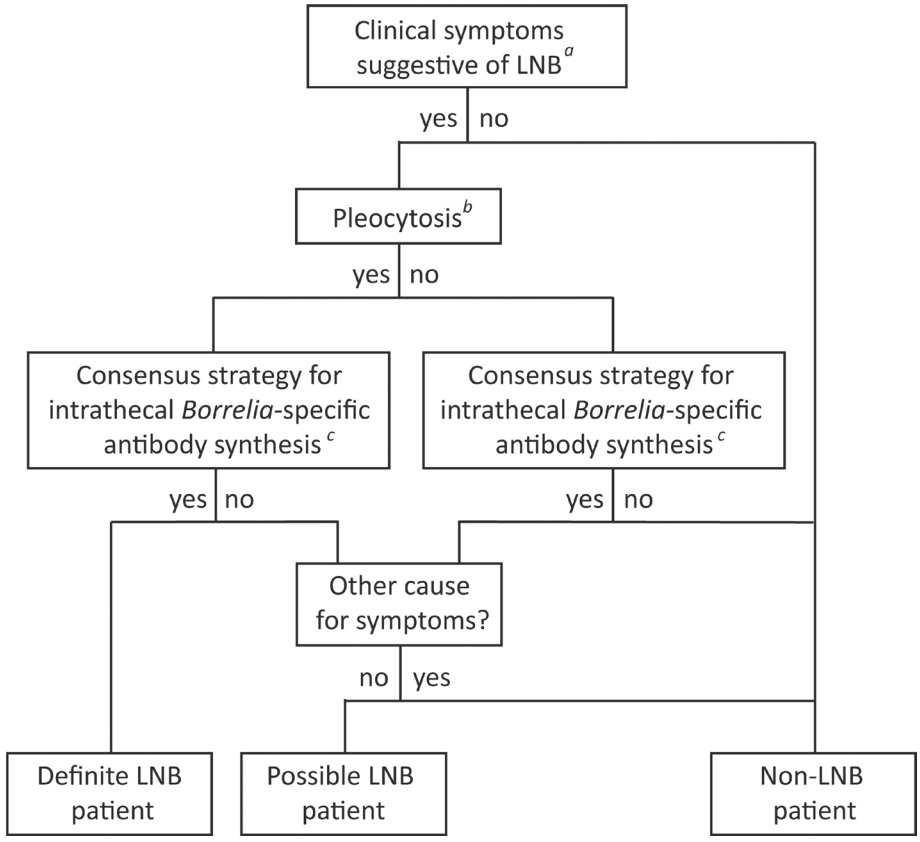

Fig. S1. Flow chart used to classify the 156 patients included in this study into definite and possible Lyme neuroborreliosis (LNB) and non-LNB patients based on the criteria defined by the European Federation of Neurological Societies (EFNS) [1] and consensus strategy for proof of intrathecal *Borrelia*-specific antibody synthesis.

a. Clinical symptoms suggestive of LNB were assumed to be present when a request for the detection of intrathecal *Borrelia*-specific antibody synthesis was done at our laboratory at the time of active disease in the past for which the IDEIA LNB IgM and IgG assay (Oxoid, Hampshire, United Kingdom) was used.
b. Pleocytosis was based on a CSF leucocyte count ≥5 leucocytes/µl.
c. The consensus strategy entailed that intrathecal *Borrelia*-specific antibody synthesis was only considered proven if the majority of the CSF-serum assays under investigation (i.e., IDEIA, Medac ELISA, *recom*Bead assay, Serion ELISA and Enzygnost ELISA) showed a pathological *Borrelia*-specific IgM and/or IgG antibody index value (≥1.5).

Table S2. The criteria for classification and the laboratory test results of the 156 patients included in this study, by study group (i.e., definite Lyme neuroborreliosis [LNB], possible LNB and non-LNB patients). Classification was done based on the criteria of the European Federation of Neurological Societies (EFNS [1]) and consensus strategy and comprised the presence of clinical symptoms suggestive of LNB without another cause, pleocytosis and intrathecal *Borrelia*-specific antibody synthesis (see also Figure S1). Intrathecal *Borrelia*-specific antibody synthesis was based on the consensus strategy, which entailed that intrathecal *Borrelia*-specific antibody synthesis was only considered proven if the majority of the cerebrospinal fluid (CSF)-serum assays under investigation showed a pathological *Borrelia*-specific IgM and/or IgG antibody index value (≥1.5). Within each study group, a row represents a unique combination of EFNS criteria and laboratory test results. Black boxes indicate the presence of the EFNS criterium or a positive laboratory test result, white boxes indicate the absence of the EFNS criterium or a negative laboratory test result. Within these boxes, M stands for an IgM response, G for an IgG response, and MG for an overall Ig (IgM and IgG) response. Grey boxes indicate missing values (ND, not determined).

					Intrathecal *Borrelia*-specific antibody synthesis																		
		EFNS criteria [1]			CSF-serum assays					CSF-only assays		Two-tier serology on serum[a]											
Study group	No. of patients[b]	Presence of clinical symptoms suggestive of LNB[c]	Pleocytosis (≥5 leucocytes/µl)	Consensus strategy[d]	IDEIA	Medac ELISA	*recom*Bead assay	Serion ELISA	Enzygnost ELISA	C6 ELISA	*recom*Line immunoblot[e]	C6 ELISA	*recom*Line immunoblot[e]	*Borrelia* species PCR on CSF	CXCL13 result on CSF	Elevated total protein in CSF	Dysfunctional blood-CSF barrier	Intrathecal total Ab synthesis (>10%)[f]	Reibergram classification[g]	Clinical symptoms / diagnosis[h]	Symptom duration[i]	Antibiotic treatment for LNB[j]
Definite LNB																							
n=10	1	■		5/5	MG	G	G	MG	MG		G		MG					MG	area 3	Radiculopathy and erythema migrans	43	ctrx 28
	1	■		5/5	G	G	MG	MG	MG		G		G				■	MG	area 3	Radiculopathy	108	ctrx 14
	1[a]	■		4/4	G	G	G	G	ND[n]		G		G				■		area 2	Radiculopathy and cognitive impairment	63	ctrx 28
	1[a]	■		4/4	G	MG	MG	G	ND[n]		G		MG			■	■	MG	area 3	Myelitis transversa	3	ctrx 14
	1[a]	■		4/4	MG	MG	MG	MG	ND[n]		G		G	■		■	■	MG	area 3	Radiculopathy and cognitive impairment	32	ctrx 14
	1	■		4/4	G	G	G	G	ND[n]		G		G						area 1	Radiculopathy	14	ctrx 14
	1[a]	■		4/4	G	G	G	MG	ND[n]		G[o]		G			■	■	MG	area 3	Polyradiculopathy with cranial (nerve VII) and peripheral neuropathy	21	ctrx 28
	1	■		3/4		G	MG	ND[m]	G		G		G			■	■	M	area 3	Radiculopathy	174	ctrx/doxy
	1	■		4/5		MG	G	MG	MG	■			MG				■	M	area 3	Cranial neuropathy (nerve VII)	22	ctrx 14
	1	■		4/5		MG	G	G	G	■		■					■		area 2	Cranial neuropathy (nerve VII) and tickbite	5	ctrx 14
Total	10	10	10	10	7	10	10	9	5	10	8[o]	10	9	2	9	6	9	7	area 1: n=1 area 2: n=2 area 3: n=7 area 4: n=0				

Possible LNB

n=4	1	■	■	2/5		M			M									M	area 3	Cranial neuropathy (nerve V)	47	ctrx 28
	1	■	■	1/4			ND[k]		M				MG						area 1	Cranial neuropathy (nerve VII)	13	ctrx 14
	1[a]	■	■	0/4					ND[a]				MG				■		area 2	Cranial neuropathy (nerve VII)	8	ctrx 14
	1[a]	■	■	0/3			ND[l]		ND[a]				G						area 1	Cranial neuropathy (nerve VI)	39	ctrx 14
n=3	1	■		5/5	MG	G	G	G	G				G					M	area 4	Peripheral neuropathy	288	doxy 30
	1	■		4/5		G	G	G	G		G°		G				■		area 2	Peroneus neuropathy	79	
	1	■		4/5		G	G	MG	G				MG					M	area 4	White matter lesions	229	
Total		7	7	4	3	1	4	3	3	5	6	1°	7	6	0	1	0	3	3	area 1: n=2 area 2: n=2 area 3: n=1 area 4: n=2		

non-LNB

n=3	1	■	■	1/5					G								■	MG	area 3	Neurosyphilis		
	1	■	■	0/4			ND[l]				■	G°	■	G					area 1	Peripheral neuropathy (isolated paralysis flexor pollicis due to a Schwannoma in shoulder)		
	1	■	■	0/5									■	G					area 1	Previously treated LNB (76 days prior)		
n=36	1	■		2/5			G		M		■	G°	■	G					area 1	General malaise, black outs, no final diagnosis made		
	1	■		1/5			G				■	G°	■	G					area 1	Acrodermatitis chronica atrophicans		
	2	■		1/5			G						■	G					area 1	Nonspecific tendon-myogenic pain (n=1); Peripheral neuropathy (n=1)		
	1	■		2/5				G	G				■	G°			■		area 1	Demyelinating condition		
	2	■		1/5				G					■	G					area 1	Peripheral neuropathy (tricheminus neuralgie) (n=1); Spinal stenosis (n=1)		
	1	■		1/5					G				■	M				M	area 4	Demyelinating condition (MS)		
	1	■		0/5							■	G°	■	G			■		area 2			
	1	■		0/5							■		■	G					area 1			
	1	■		0/5									■	G			■	■	area 2			
	1	■		0/5								G°	■	G			■		area 2			
	1	■		0/5									■	M					area 1			
	12	■		0/5									■	G					area 1			
	3	■		0/5									■	MG					area 1			
	1	■		0/5									■				■		area 2			
	1	■		0/5									■	G°					area 1			
	1	■		0/5															area 1			
	5	■		0/5									■						area 1			
n=19	1		■	0/4				ND[m]							■		■		area 2	Tuberculous meningitis		
	1		■	0/5													■		area 2			
	1		■	0/5										M[p]			■		area 2			
	3		■	0/5											■		■		area 2			
	1		■	0/5														M	area 4			
	3		■	0/5														G	area 4			
	1		■	0/5														MG	area 4			
	8		■	0/5															area 1			

Table S2. Continue.

		EFNS criteria [1]			Intrathecal *Borrelia*-specific antibody synthesis							Two-tier serology on serum[a]										
					CSF-serum assays					CSF-only assays												
Study group	No. of patients[b]	Presence of clinical symptoms suggestive of LNB[c]	Pleocytosis (≥5 leucocytes/µl)	Consensus strategy[d]	IDEIA	Medac ELISA	*recom*Bead assay	Serion ELISA	Enzygnost ELISA	C6 ELISA	*recom*Line immunoblot[e]	C6 ELISA	*recom*Line immunoblot[e]	*Borrelia* species PCR on CSF	CXCL13 result on CSF	Elevated total protein in CSF	Dysfunctional blood-CSF barrier	Intrathecal total Ab synthesis (>10%)[f]	Reibergram classification[g]	Clinical symptoms / diagnosis[h]	Symptom duration[i]	Antibiotic treatment for LNB[j]
n=81	1			1/5				G										G	area 4	Treated neurosyphilis		
	2			1/5				G											area 1	Panic disorder (n=1); Radiculopathy (n=1)		
	1			0/5									M[p]				■		area 2			
	7			0/5													■		area 2			
	3			0/5												■	■		area 2			
	3			0/5														M	area 4			
	3			0/5														G	area 4			
	1			0/5								■							area 1			
	2			0/5									M[p]						area 1			
	58			0/5															area 1			
Total	139	39	22	0	0	0	4	6	4	5	5[o]	38	31[o] (+4[p])	0	2	10	22	14	area 1: n=104 area 2: n=21 area 3: n=1 area 4: n=13			
Total (all patients)	156	56	36	13	8	14	17	18	14	21	14[o]	55	46[o] (+4[p])	2	12	16	34	24	area 1: n=107 area 2: n=25 area 3: n=9 area 4: n=15			

a. Two-tier serology on serum was performed using the C6 ELISA as a screening test, and positive (and equivocal) C6 ELISA results were confirmed using the *recom*Line IgM and IgG immunoblot.
b. One hundred and fifty (96.2%) of the 156 study participants were consecutive patients who were eligible for inclusion if a CSF and a blood sample (drawn within 24 h of the lumbar puncture) had been sent to the microbiology laboratory of the Diakonessenhuis Hospital, Utrecht, the Netherlands, in the period between August 2013 and June 2016. Six (3.8%) of the 156 study participants (four definite and two possible LNB patients; all of them are marked with 'a' in the table) were selected from outside this period (from February 2011 to July 2013 and from July 2016 to November 2017).
c. Clinical symptoms suggestive of LNB were assumed to be present when a request for LNB diagnostics was done at the time of active disease in the past, which included the detection of intrathecally-produced *Borrelia*-specific IgM and IgG using the IDEIA.
d. The consensus strategy entailed that intrathecal *Borrelia*-specific antibody synthesis was only considered proven if the majority of the CSF-serum assays under investigation (i.e., IDEIA, Medac ELISA, *recom*Bead assay, Serion ELISA and Enzygnost ELISA) showed a pathological *Borrelia*-specific IgM and/or IgG antibody index value (≥1.5).
e. The IgG results are based on the revised interpretation criteria of the *recom*Line IgG immunoblot implemented by the manufacturer in January 2019 (Table S3).
f. Intrathecal total antibody (Ab) synthesis is proven if the intrathecal total IgM and/or total IgG fraction is larger than 10% as described by Reiber [2].
g. The Reibergram classification is based on the blood-CSF barrier functionality and the presence of intrathecal total antibody (IgM and/or IgG) synthesis [2]. The four areas listed in this column are

explained as follows: area 1, a normal blood-CSF barrier without intrathecal total-antibody synthesis; area 2, a dysfunctional blood-CSF barrier without intrathecal total-antibody synthesis; area 3, a dysfunctional blood-CSF barrier with intrathecal total-antibody synthesis; and area 4, a normal blood-CSF barrier with intrathecal total-antibody synthesis, the results of which have been published previously [3].

h. Clinical symptoms for patients who had a pathological IgM and/or IgG AI value in at least one of the five CSF-serum assays, and/or a positive test result in at least one of the two CSF-only assays, and/or a positive *Borrelia* species PCR result on CSF, and/or a positive CSF-CXCL13 result are listed in the corresponding row and if this included patients for whom LNB was ruled out, the alternative diagnosis was shown. For all other (non-LNB) patients, diagnoses that were found at least twice included peripheral neuropathy (*n* = 18), demyelinating conditions (*n* = 15) including six cases of multiple sclerosis (MS), radiculopathy (*n* = 8), non-CSF infectious disease (*n* = 7), spinal stenosis (*n* = 6), (transient) facial nerve paralysis (*n* = 6), proven non-LNB CSF infectious disease (*n* = 3; streptococcus (*n* = 1) and viral (*n* = 2) meningitis); nonspecific tendon-myogenic pain (*n* = 3), cerebrovascular accident (*n* = 3), headache/migraine (*n* = 3), cancer (*n* = 3), epilepsy (*n* = 2), sleep disorder (*n* = 2), psychogenic disorder (*n* = 2), microvascular white matter lesions (*n* = 2), and arthralgia (*n* = 2). A unique diagnosis was found for 18 non-LNB patients and 21 non-LNB patients never received a diagnosis (data not shown).

i. Symptom duration is the number of days between the start of symptoms and the lumbar puncture and is only shown for definite and possible LNB patients.

j. Of the 17 patients that were classified as definite or possible LNB patient, 15 (10 definite and 5 possible LNB patients) had been treated for LNB following the recommendations of the Dutch guidelines for LB [4]. This treatment had started after the LP was performed with a median of 0 days [range 0-18] after CSF-blood sampling. Nine of the 10 definite LNB patients were treated with ceftriaxone (2 g/day) intravenously for either 14 (*n* = 6; [ctrx 14]) or 28 (*n* = 3; [ctrx 28]) days. One definite LNB patient had started with intravenous ceftriaxone (2 g/day), but after 5 days switched to oral doxycycline (100 mg twice a day) for 25 days because of an allergic reaction (ctrx/doxy). Four of the five possible LNB patients received ceftriaxone (2 g/day) intravenously for either 14 (*n* = 3; [ctrx 14]) or 28 (*n* = 1; [ctrx 28]) days. The remaining possible-LNB patient received oral doxycycline (100 mg twice a day) from the start for 30 days (doxy 30).

k. For one possible LNB patient, the IgM antibody index value using the *recom*Bead assay is missing due to insufficient sample material (for this patient, the IgG antibody index value using the *recom*Bead assay is normal).

l. For two cases, one possible LNB and one non-LNB patient, the IgG antibody index value using the *recom*Bead assay is missing due to insufficient sample material (for these patients, the IgM antibody index value using the *recom*Bead assay is normal).

m. For two cases, one definite LNB and one non-LNB patient, the IgM and IgG antibody index values using the Serion ELISA were not determined due to insufficient sample material.

n. For seven cases, five definite and two possible LNB patients, the IgM and IgG antibody index values using the Enzygnost ELISA were not determined as the ELISA was taken of the market.

o. The positive overall Ig *recom*Line immunoblot result was based on a negative result for IgM and a positive result for IgG. For IgG, this result was based on the revised interpretation criteria of the *recom*Line IgG immunoblot (Table S3); however, using the old interpretation criteria, the IgG result on CSF for seven cases (one definite LNB, one possible LNB and five non-LNB patients) and the IgG result on serum for two non-LNB patients, was negative. Consequently, the overall Ig result based on the old interpretation criteria of the *recom*Line IgG immunoblot would also have been negative for these cases.

p. The *recom*Line immunoblot was tested on all 156 sera and the results were compared with those obtained in CSF to assess the origin of the *Borrelia*-specific antibodies (CSF or blood-derived). For four non-LNB patients, all of whom had a negative C6 ELISA result on serum and were, thus, considered seronegative using the two-tier testing algorithm, the *recom*Line IgM immunoblot on serum was positive. For three of them, IgM *recom*Line immunoblot positivity was based on a positive band for either one (*n* = 2) or three (*n* = 1) OspC antigens. For one non-LNB patient, IgM *recom*Line immunoblot positivity was based on a positive band for p41 and p18 *B. garinii*.

Table S3. Test characteristics of the five antibody assays tested on CSF-serum pairs (A) and the two antibody assays tested on CSF only (B) used for the detection of intrathecally produced *Borrelia*-specific antibodies

	Assays	Technique	Target	Quantification	Intrathecal antibody synthesis	Normal AI value	Borderline AI value	Pathological AI value
A.	IDEIA™ Lyme Neuroborreliosis	Capture ELISA	IgM and IgG: purified native DK1 flagellum (p41) *B. afzelii*	Semi-quantitative; OD	AI by using the formula: (OD CSF/ OD SER) * (OD CSF - OD SER)	AI < 0.3 or OD CSF <0.150	NA	AI ≥ 0.3
	Borrelia-IgM-ELISA Medac *Borrelia*-IgG-ELISA Medac	ELISA	IgM: OspC and VlsE IgG: VlsE	Quantitative (concentration using single-point quantification with lot-specific calibration curve)	AI by Reiber and Peter [8]	0.6 ≤ AI ≤ 1.3	1.3 < AI ≤ 1.5[f]	AI > 1.5
	*recom*Bead Borrelia IgM 2.0 *recom*Bead Borrelia IgG 2.0	Luminex	IgM and IgG against highly purified recombinant antigens from *Bbss, Bafz, Bgar, Bbav,*and *Bsp*. Different fluorescent-labelled beads are coated with either one of the following antigens: p100, VlsE, p58, p39, OspA, OspC (*Bbss, Bafz, Bgar*)[a], and p18 (*Bbss, Bafz, Bbav, Bgar, Bsp*)[a,b]	Semi-quantitative; MFI	For each antigen separately, an AI is calculated by Reiber and Peter [8]. A single pathological AI value is sufficient to suggest the intrathecal synthesis of *Borrelia*-specific antibodies	0.6 ≤ AI ≤ 1.3	1.3 < AI < 1.5[f]	AI ≥ 1.5
	Borrelia burgdorferi IgM SERION ELISA classic Borrelia burgdorferi IgG SERION ELISA classic	ELISA	IgM: whole-cell lysates *B. afzelii* Pko [5] and *B. garinii* [6] IgG: whole-cell lysates *B. afzelii Pko* [5] *and B. garinii* [6] + recombinant VlsE	Quantitative (concentration using single-point calibration with 4-PL method and lot-specific calibration curve)	AI by Reiber and Peter [8]	0.7 ≤ AI ≤ 1.4	NA	AI > 1.5
	Enzygnost® Borreliosis/IgM Enzygnost® Lyme link VlsE/IgG	ELISA	IgM: whole-cell lysate *B. burgdorferi* PKo IgG: whole-cell lysate *B. afzelii* PKo + recombinant VlsE	Quantitative (OD index for IgM and concentration using the alfa-method for IgG)	AI by Reiber and Peter [8]	0.5 ≤ AI ≤ 1.49	NA	AI ≥ 1.5
B.	C6 ELISA	ELISA	Total Ig (IgM and IgG) against synthetic C6 peptide, derived from a highly immunogenic part (invariable region 6) of the VlsE lipoprotein [7]	Semi-quantitative by Lyme Index[c]	NA	NA	NA	NA
	*recom*Line IgM and IgG [9,10][d]	Immunoblot	IgM and IgG against various antigens from *Bbss, Bafz, Bgar, Bbav,* and *Bsp.* Antigens coated are p100, VlsE, p58, p41, p39, OspA, OspC (*Bbss, Bafz, Bgar, Bsp*)[a], and p18 (*Bbss, Bafz, Bbav, Bgar2, Bsp*)[a,b]	Qualitative by intensity and number of bands[d,e]	NA	NA	NA	NA

AI, antibody index; Ig, immunoglobulin; OD, optical density; CSF, cerebrospinal fluid; SER, serum; NA, not applicable; Osp, outer surface protein; VlsE, variable major protein-like sequence, expressed; *Bbss*, *B. burgdorferi* sensu strictu; *Bafz*, *B. afzelii*, *Bgar*, *B. garinii*; *Bbav*, *B. bavariensis*; *Bsp*, *B. spielmanii*; MFI, median fluorescence intensity; 4-PL, 4-parameter logistic

a. Reactivity against multiple OspC antigens is counted only once, irrespective of how many OspC antigens react. This also applies for reactivity against multiple p18 antigens.
b. p18 = DbpA, decorin binding protein.
c. For both CSF and serum: C6 ELISA results were interpreted by the calculation of a Lyme index (LI) for which the following formula was used: LI = OD sample / (OD calibrator + 0.3) as described in the instruction manual of the manufacturer for serum. Subsequently, results were classified as either negative (LI ≤ 0.90), equivocal (0.91 < LI < 1.09), or positive (LI ≥ 1.10).
d. The manufacturer of the *recom*Line immunoblot revised the interpretation of the *recom*Line IgG immunoblot in January 2019 by increasing the point value of the VlsE band from 5 to 6, which has an effect on the test result. In the current study, both the old and the revised interpretation criteria were used; however, only the revised interpretation criteria were elaborated on in the manuscript.
e. For both CSF and serum: point values for IgM-bands: p100 = 5, VlsE = 5, p58 = 4, p41 (flagellin) = 1, p39 = 4, OspA = 5, OspC = 8, p18 (DbpA) = 5. Point values for IgG-bands: p100 = 5, VlsE = 5, p58 = 4, p41 (flagellin) = 1, p39 = 5, OspA = 5, OspC = 5, p18 (DbpA) = 5. Results are based on the sum of the point values of the bands that have an equal or larger intensity than the cutoff band and are interpreted as negative (≤5 points), equivocal (6 points), or positive (≥7 points).
f. AI values that were equivocal should have been repeated; however, this was not done due to limited sample material.

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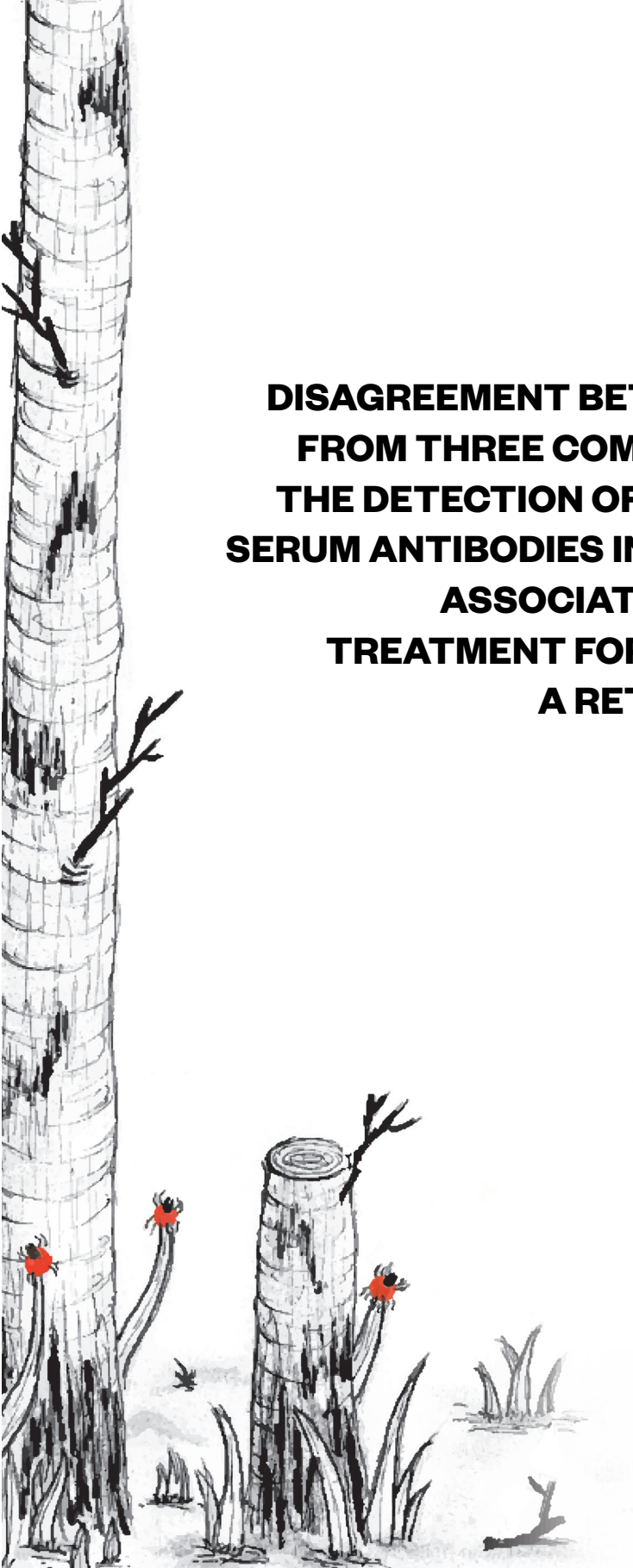

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DISAGREEMENT BETWEEN THE RESULTS FROM THREE COMMERCIAL TESTS FOR THE DETECTION OF *BORRELIA*-SPECIFIC SERUM ANTIBODIES IN THE NETHERLANDS ASSOCIATED WITH ANTIBIOTIC TREATMENT FOR LYME BORRELIOSIS: A RETROSPECTIVE STUDY

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ABSTRACT

The diagnosis of Lyme borreliosis is challenging because of the often non-specific symptoms and persisting antibodies after infection. We investigated the diagnostic characteristics of two enzyme-linked immunosorbent assays (ELISAs) and an immunoblot for the detection of *Borrelia*-specific serum antibodies using different test strategies in individuals with and without antibiotic treatment for Lyme borreliosis. This retrospective study included healthy individuals, patients with active Lyme neuroborreliosis and patients treated for Lyme neuroborreliosis. Two ELISAs were compared: the C6 ELISA and the Serion ELISA. Equivocal and positive results were confirmed by immunoblot. We included 174 healthy individuals, of whom 27 (15.5%) were treated for Lyme borreliosis in the past, 36 patients were treated for Lyme neuroborreliosis and 27 patients had active Lyme neuroborreliosis. All the active Lyme neuroborreliosis patients were reactive in both ELISAs (100% sensitivity); less reactivity was seen in the other three groups (range 17.7% to 69.4%). The concordance between the ELISA results was high in active Lyme neuroborreliosis patients (26/27; 96.3%) and healthy individuals (131/147; 89.1%), but lower in treated healthy individuals (18/27; 66.7%) and treated Lyme neuroborreliosis patients (18/36; 50.0%) ($P \leq 0.005$). This study showed that antibiotic treatment against Lyme borreliosis was strongly associated with discordant ELISA and test strategy results (odds ratio: 10.52; $P < 0.001$ and 9.98; $P = 0.014$, respectively) suggesting antibiotic treatment influences the pace at which the various antibodies directed to the different antigens used in both ELISAs wane. Among treated neuroborreliosis patients, the Serion ELISA stayed positive for a longer period after infection compared to the C6 ELISA. This should be taken into consideration when requesting and/or interpreting Lyme serology.

INTRODUCTION

The recommended approach for the diagnosis of Lyme borreliosis consists of screening for *Borrelia*-specific serum antibodies with an enzyme-linked immunosorbent assay (ELISA), followed by immunoblot confirmation of equivocal or positive ELISA results [1]. The reliability of the serodiagnosis of Lyme borreliosis is influenced by various factors, including the manifestation and the duration of disease, the natural clearance of infection, antibiotic treatment, (age-specific) seroprevalence and the test characteristics, such as the antigens used [2]. Antibiotic therapy can abrogate the immune response, but the persistence of *Borrelia*-specific serum antibodies up to several years after antibiotic treatment has also been reported [3, 4]. In the Dutch population, the seroprevalence is 4–8%, but is higher in certain risk groups, such as forestry workers (20%) [5, 6].These seropositive cases are usually asymptomatic, suggesting a cleared infection with the persistence of *Borrelia*-specific serum antibodies.

A large variety of diagnostic assays for Lyme borreliosis is available. Some assays make use of whole-cell lysates, which are mostly derived from cultured *Borrelia burgdorferi* sensu stricto, *Borrelia afzelii* or *Borrelia garinii* [7, 8]. These assays have a potential problem of higher cross-reactivity with common antigens of other micro-organisms [9]. Recombinant antigens, such as OspC, DbpA [10, 11] and especially VlsE (Vmp-like sequence) and C6 peptide are more specific [12, 13]. Although studies have compared different assays using different test strategies, these studies lacked well-defined study populations [14].

Therefore, we used well-described patient groups as well as healthy individuals to compare two standard two-tier test strategies, based on an ELISA (either the C6 ELISA or the Serion ELISA), followed by immunoblot confirmation of equivocal and positive ELISA results. The C6 ELISA measures total immunoglobulin to a recombinant C6 peptide and is currently used in our laboratory. The Serion ELISA measures IgM and IgG to two whole-cell lysates of *B. burgdorferi* sensu lato. It is an improved version compared to the one used by Smismans et al. [8] by the addition of recombinant VlsE for the detection of IgG. The Serion ELISA was selected because it is based on different antigens and it uses VlsE instead of the C6 peptide. VlsE evokes a different antibody response compared to the C6 peptide, since the C6 peptide only becomes available after a conformational change of VlsE when *Borrelia* enters the human body [12, 15]. A third test strategy was also included and consisted of a more unconventional approach based on the combination of both ELISAs as a screening test and immunoblot confirmation of all results, except concordant negative results.

MATERIALS AND METHODS

STUDY POPULATION

To qualify for inclusion in this study, all healthy individuals and (hospital) patients had to be ≥18 years old. Healthy individuals, with an increased risk of a tick bite, were recruited in the period between February 2013 and December 2015. Most healthy individuals consisted of personnel of the Diakonessenhuis Hospital or the St. Antonius Hospital, both located in the center of the Netherlands, close to forested areas. In the same period, Boy Scout patrol leaders, owners of hunting dogs, recreational runners and personnel of the National Institute for Public Health and the Environment (RIVM), all with recreational activities in high-risk areas for tick bites, were asked to participate. Healthy individuals who mentioned antibiotic treatment for Lyme borreliosis in the past in their questionnaire were included in a separate group, referred to as treated healthy individuals. Lyme neuroborreliosis patients enrolled in this study had to fulfil at least two of the following criteria, as proposed by the European Federation of Neurological Societies (EFNS)

[16]: (i) the presence of neurological symptoms suggestive of Lyme neuroborreliosis without other obvious explanations, (ii) cerebrospinal fluid (CSF) pleocytosis (≥5 leucocytes/µl) and/or (iii) *Borrelia*-specific intrathecal antibody production. A patient was diagnosed with definite Lyme neuroborreliosis when all three criteria were met and with possible Lyme neuroborreliosis when two criteria were fulfilled and they had either been treated for Lyme neuroborreliosis in the past or were recently diagnosed with (active) Lyme neuroborreliosis. For inclusion in the study, intrathecal IgM/IgG was determined by the second generation IDEIA™ Lyme Neuroborreliosis test (Oxoid, Cambridgeshire, United Kingdom). Patients who had been diagnosed and treated for Lyme neuroborreliosis between February 2004 and September 2012 were enrolled from March 2013 to March 2015; active Lyme neuroborreliosis patients were recruited from December 2010 to December 2015 and were only included if they had not yet started antibiotic treatment for Lyme neuroborreliosis. Active Lyme neuroborreliosis patients who had finished antibiotic treatment could also be included as treated Lyme neuroborreliosis patients when at least one year had passed after their inclusion as an active Lyme neuroborreliosis case.

All healthy individuals and patients were asked to fill in a Lyme-specific questionnaire, which included questions regarding tick bites, presence of erythema migrans (EM), antibiotic treatment for Lyme borreliosis and (self-reported) complaints at the moment of inclusion and during possible earlier episodes of Lyme borreliosis. Information regarding the clinical symptoms, pleocytosis, intrathecal antibody production and the clinical outcome (in case of treated Lyme neuroborreliosis patients) was extracted from the hospital information system. Patients were considered to have a good recovery when, within six months after antibiotic treatment finished, symptoms were absent or had considerably decreased. Patients were considered as treatment failure if severe symptoms continued for >6 months after they had finished antibiotic treatment. Healthy individuals were only recruited when they reported no complaints at the moment of inclusion.

SEROLOGY

Serum samples of all study subjects were tested in two ELISAs and one immunoblot. All tests were performed according to the manufacturer's instructions using a DS2 automated ELISA instrument (Dynex® DS2, Dynex Technologies, Chantilly, VA, USA) and analyzed with the DS-Matrix™ software (Dynex Technologies). An ELISA result was called reactive when the result was equivocal or positive [5]. When both IgG and IgM were determined separately (Serion ELISA and immunoblot), the final 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 ELISA result. The C6 ELISA was performed immediately after blood sampling. Immunoblot confirmation was done ≤2 weeks after blood sampling for samples with C6 ELISA index scores between 0.91 to 3.00, according to the protocol of our laboratory. For the purpose of this study, the Serion ELISA was performed on all samples several months to years later. In addition, the samples with C6 ELISA index scores ≥3.00 and any additional reactive Serion ELISA/negative C6 ELISA results were confirmed with immunoblot. Blood samples were stored at +4°C for two weeks after blood sampling and at −20°C for longer storage.

C6 ELISA

The C6 ELISA (Immunetics, Boston, MA, USA) is based on a synthetic C6 peptide, which is derived from a highly immunogenic part (invariable region 6) of the VlsE lipoprotein [13].

SERION ELISA

The SERION ELISA IgM and IgG tests (Serion ELISA classic *Borrelia burgdorferi* IgM and IgG,

Institute Virion/ Serion GmbH, Würzburg, Germany) are both based on a combination of bacterial lysates of *B. afzelii* PKo [17] and *B. garinii* [18]. For IgG detection, the lysates are enriched with recombinant VlsE.

IMMUNOBLOT

*Recom*Line IgM and IgG strips (Mikrogen GmbH, Neuried, Germany) containing purified recombinant *B. burgdorferi* sensu lato antigens (OspA, OspC, p100, VlsE, p39, p58 and p18) were used [19]. The results were measured with an automated *recom*Scan system using the *recom*Scan Software (Mikrogen GmbH).

TEST STRATEGIES

In this study, three different test strategies were compared. The first two strategies were based on the recommended two-tier test strategy; a screening ELISA followed by immunoblot (IB) confirmation of equivocal or positive ELISA results [1]. The C6 ELISA was used as a screening assay in the first strategy (C6/IB strategy) and the second strategy used the Serion ELISA as a screening test (SE/IB strategy). The third strategy was based on the two ELISAs followed by immunoblot confirmation on all combinations of results, except concordant negative results (SE + C6/IB strategy).

STATISTICAL ANALYSIS

Dichotomous, unrelated samples were analyzed using Pearson's Chi-squared test or Fisher's exact test. *Post-hoc* tests consisted of two-group comparisons using Pearson's Chi-squared test or Fisher's exact test. The non-parametric Cochran's Q test for >2 related samples was used for comparison of the ELISA and strategy results and *post-hoc* tests consisted of the McNemar's test. For these statistical analyses, equivocal results were combined with positive results. Quantitative data were analyzed using the Kruskal–Wallis test for >2 group comparisons. Two-group comparisons and *post-hoc* tests consisted of the Mann–Whitney test. A P value <0.05 was interpreted as statistically significant, unless Bonferroni correction was applied; Bonferroni correction was applied to all *post-hoc* tests. Concordance was determined between the C6 ELISA and the Serion ELISA and between the C6/IB strategy and the SE/IB strategy; concordance was calculated as the number of matching positive, equivocal and negative results compared to the overall results within a group. Correlations were calculated using Spearman's correlation coefficient (r_s). Logistic regression was applied to calculate the contribution of various variables that could cause discordant test results. The IBM SPSS software package (version 21) was used (Armonk, NY, USA).

RESULTS

STUDY POPULATION

(TREATED) HEALTHY INDIVIDUALS

A total of 174 healthy individuals were included in this study and the median age at inclusion was 42.3 years (interquartile range (IQR): 28.0–53.4). Twenty-seven of 174 (15.5%) healthy individuals reported antibiotic treatment for Lyme borreliosis in the past (median of 5.0 years ago; IQR: 2.0–7.0) and were, therefore, classified as treated healthy individuals; 22/27 (81.5%) reported having had an EM, 4/27 (14.8%) reported a diffuse redness after a tick bite and 1/27 (3.7%) had flu-like symptoms after a tick bite (Table 1). The median age at inclusion of the treated healthy individuals was 53.1 years (IQR: 38.3–57.6). The remaining 147/174 (84.5%) individuals were classified as healthy individuals and had a median age of 40.9 years (IQR: 27.0–51.8) at inclusion and were younger than the other three groups ($P \leq 0.001$) (Table 1).

TREATED LYME NEUROBORRELIOSIS PATIENTS

Thirty-six treated Lyme neuroborreliosis patients were included and their median age was 59.1 years (IQR: 49.4–66.2) (Table 1). Most treated Lyme neuroborreliosis patients had been diagnosed with radiculopathy (*n* = 24; 66.7%) or cranial nerve paresis (*n* = 17; 47.2%), such as facial nerve paralysis (nerve VII) or abducens nerve palsy (nerve VI). Other diagnoses included meningitis (*n* = 2; 5.6%) and peripheral neuropathy (*n* = 3; 8.3%). Seven patients with radiculopathy also suffered from facial nerve paralysis, one also had meningitis. One patient had been diagnosed with facial nerve paralysis and peripheral neuropathy. Treated Lyme neuroborreliosis patients were included approximately 6.1 years (IQR: 3.5–8.4) after antibiotic treatment finished and treatment consisted of intravenous ceftriaxone for 14 or 30 days; however, one patient switched to doxycycline (for 14 days) after 10 days due to an allergic reaction. As many as 33/36 (91.7%) of the treated Lyme neuroborreliosis patients had a known clinical outcome; 27 (81.8%) of them had a good recovery and six (18.2%) of them were considered a treatment failure (Table 1).

Table 1. Demographic and clinical characteristics of the four different groups in this study

Variable	Healthy individuals (*n* = 147)	Treated healthy individuals (*n* = 27)	Treated LNB patients[b] (*n* = 36)	Active LNB patients[b] (*n* = 27)	*P* value (overall)	*P* value (2-group[d])
Sex; no. of males (%)	57 (38.8)	12 (44.4)	19 (52.8)	14 (51.9)	0.339	NA
Age (at inclusion); median, years (IQR)	40.9 (27.0–51.8)	53.1 (38.3–57.6)	59.1 (49.4–66.2)	57.8 (47.8–72.8)	<0.001	≤0.001[e]
Tick bite (%)	86 (58.5)	25 (92.6)	27 (75.0)	9 (56.3)[c]	0.003	≤0.008[f]
EM (%)	4 (2.7)	22 (81.5)	9 (25.0)	4 (25.0)[c]	<0.001	≤0.004[g]
Time of inclusion after AB treatment finished; median, years (IQR)	NA	5.0 (2.0–7.0)[a]	6.1 (3.5–8.4)	NA	NA	0.071
Pleocytosis (before AB treatment started); median (IQR)	NA	NA	52.0 (21.0–113.5)	112.0 (33.0–214.0)	NA	0.050
Definite LNB; *n* (%)	NA	NA	30 (83.3)	22 (81.5)	NA	0.553
Possible LNB based on clinical symptoms and:	NA	NA				
Pleocytosis; *n* (%)			1 (2.8)	4 (14.8)		
Intrathecal *Borrelia*-specific antibody production; *n* (%)			5 (13.9)	1 (3.7)		
Recovery status[h]						
Good recovery; *n* (%)			27 (81.8)			
Treatment failure; *n* (%)			6 (18.2)			

LNB, Lyme neuroborreliosis; EM, erythema migrans; IQR, interquartile range; AB, antibiotic; NA, not applicable

a. One treated healthy individual who did not know when antibiotic treatment took place was excluded.
b. Eight active Lyme neuroborreliosis patients were also included as treated Lyme neuroborreliosis patients.
c. Eleven active Lyme neuroborreliosis patients did not fill out the Lyme-specific questionnaire and were, thus, excluded from the calculations.
d. For all two-group comparisons with a significant difference, the Bonferroni correction was applied (*P*: 0.050/6 = 0.008)
e. Significant difference in age at inclusion for healthy individuals compared to the remaining three groups.
f. Significant difference in percentage of tick bites for treated healthy individuals compared to healthy individuals and active Lyme neuroborreliosis patients.
g. Significant difference in percentage of erythema migrans for healthy individuals compared to the remaining three groups and for treated healthy individuals compared to treated and active Lyme neuroborreliosis patients.
h. The clinical outcome could not be established for three treated Lyme neuroborreliosis patients.

ACTIVE LYME NEUROBORRELIOSIS PATIENTS

Twenty-seven active Lyme neuroborreliosis patients were included and their median age was 57.8 years (IQR: 47.8–72.8) (Table 1). Most active Lyme neuroborreliosis patients were diagnosed with radiculopathy (*n* = 8; 29.6%) or cranial nerve paresis (*n* = 7; 25.9%), such as facial nerve paralysis or abducens nerve palsy. Other diagnoses included meningitis (*n* = 5; 18.5%) or peripheral neuropathy (*n* = 2; 7.4%). Four active Lyme neuroborreliosis patients with radiculopathy also suffered from facial nerve paralysis, one patient had facial nerve paralysis and meningitis, and one patient had radiculopathy as well as peripheral neuropathy. Eight of 27 (29.6%) active Lyme neuroborreliosis patients were, at a later moment, also included as a treated Lyme neuroborreliosis patient and were, thus, included in both groups. The median time between the two blood sampling moments (and, thus, the time between their inclusion as an active and a treated case) was years (IQR: 1.4–2.5). Hence, in total, 63 blood samples originating from 55 Lyme neuroborreliosis patients were investigated in this study.

ELISA RESULTS

All 27 (100%) active Lyme neuroborreliosis patients had reactive results for both ELISAs, but the ELISA results of the other three groups showed more diversity (Fig. 1). In these three groups, the Serion ELISA resulted in more reactive cases compared to the C6 ELISA (24 vs. 15), but the difference within each group was not statistically significant (Fig. 2). The percentage of reactive ELISA results, when both ELISAs were combined, was 23.8% (35/147) for healthy individuals, 44.4% (12/27) for treated healthy individuals and 69.4% (25/36) for treated Lyme neuroborreliosis patients (Fig. 2). For both healthy individuals and treated Lyme neuroborreliosis patients, this percentage was significantly higher than the percentage of reactive C6 ELISA results alone (26/147 (17.7%) and 16/36 (44.5%), respectively) ($P \leq 0.004$ for both) (Figs. 1 and 2). For treated Lyme neuroborreliosis patients, the percentage of reactive results of the C6 ELISA and the Serion ELISA combined (25/36; 69.4%) was also significantly higher than the percentage of reactive results of the Serion ELISA alone (18/36; 50.0%) ($P = 0.016$) (Figs. 1 and 2).

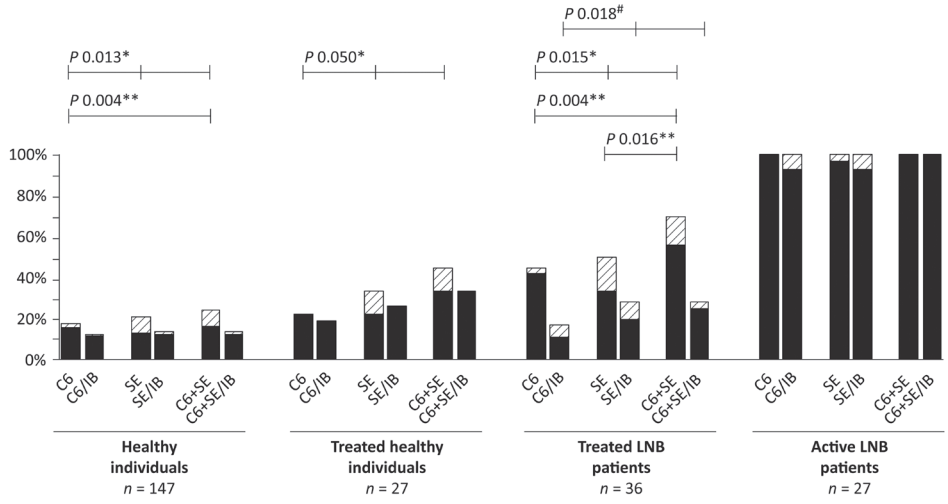

Fig. 1. Overall view of the proportions of reactive (equivocal and positive) enzyme-linked immunosorbent assay (ELISA) and test strategy results. LNB, Lyme neuroborreliosis. The black bars represent positive results and the shaded bars represent equivocal results. C6, C6 ELISA; C6/IB, C6 ELISA plus immunoblot strategy; SE, Serion ELISA; SE/IB, Serion ELISA plus immunoblot strategy; SE + C6, combination of Serion ELISA and C6 ELISA; SE + C6/IB, combination of Serion ELISA and C6 ELISA plus immunoblot strategy. The C6 + SE bars represent the percentage of reactive results for at least one of the tests (i.e., C6 ELISA, Serion IgM ELISA or Serion IgG ELISA). Only statistical significant differences are displayed. */#: Significant difference between the ELISAs (*) and the test strategies (#) in the overall dataset; **: Significant difference between the two-group comparisons of the ELISAs. For all two-group comparisons with a significant difference, the Bonferroni correction was applied ($P = (0.050/3) = 0.017$).

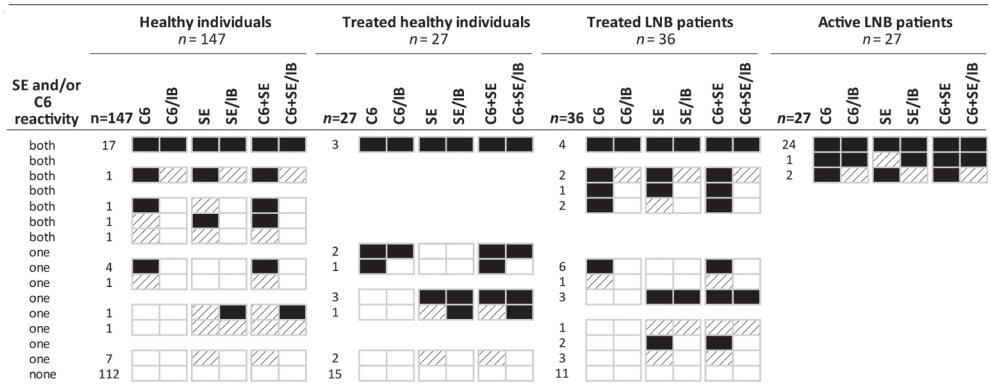

Fig. 2. Detailed view of the different ELISA and test strategy results. LNB, Lyme neuroborreliosis. The black boxes represent positive results, the shaded boxes represent equivocal results and the white boxes represent negative results. C6, C6 ELISA; C6/IB, C6 ELISA plus immunoblot strategy; SE, Serion ELISA; SE/IB, Serion ELISA plus immunoblot strategy; SE + C6, combination of Serion ELISA and C6 ELISA; SE + C6/IB, combination of Serion ELISA and C6 ELISA plus immunoblot strategy.

CONCORDANCE OF ELISA RESULTS

The concordance between both ELISAs was lower for treated healthy individuals (18/27; 66.7%) and treated Lyme neuroborreliosis patients (18/36; 50.0%) than for healthy individuals (131/147; 89.1%) and active Lyme neuroborreliosis patients (26/27; 96.3%) ($P \leq 0.005$) (Fig. 3a). The majority of the concordant ELISA results were based on negative ELISA results, except for active Lyme neuroborreliosis patients, for whom only reactive ELISA results were found. In contrast, 112/131 (85.5%) ELISA results among healthy individuals were concordant negative (Fig. 2), of whom only three (2.3%) reported a previous (untreated) EM (data not shown). Although it was unclear whether healthy individuals had been infected with *Borrelia* in the past, the percentage of concordant negative results among healthy individuals did not differ from the percentage of concordant negative results among treated healthy individuals or treated Lyme neuroborreliosis patients (15/18 (83.3%) and 11/18 (61.1%), respectively) (Fig. 2).

IMMUNOBLOT RESULTS

In total, 99 immunoblots had to be performed based on 44 (44.4%) discordant and 55 (55.6%) concordant ELISA results (Fig. 2). All 27 (10 0%) active Lyme neuroborreliosis patients were confirmed by immunoblot analysis. A lower percentage of confirmed cases was found among the other three groups. Of the 35 healthy individuals that had a reaction with one or both ELISAs, 20 (57.1%) were confirmed by immunoblot; among treated healthy individuals, this was 75.0% (9/12) and among treated Lyme neuroborreliosis patients, it was 40.0% (10/ 25) (Fig. 2).

With regard to the 55 concordant reactive ELISA results, 53 (96.4%) were confirmed by immunoblot. All 53 had a positive result for the C6 ELISA and the Serion IgG ELISA, of which 31 (58.5%) also had an equivocal or positive result for the Serion IgM ELISA. Immunoblot confirmation was based on an IgG response against at least two antigens; IgG against VlsE was found in all 53 cases, followed by IgG against p41 flagellin (39/53; 73.6%) and subsequently by IgG against p100, p18, p58, p39 and OspC (range 23 to 9; 43.3% to 17.0%) (data not shown). Only 16/53 (30.2%) cases were also confirmed based on the presence of IgM, of which most cases were found among active Lyme neuroborreliosis patients (13/16; 81.3%). For all 16 IgM confirmed cases, antibodies were found against OspC, followed by IgM against p41 flagellin (11/16; 68.8%) (data not shown). The remaining 2/55 (3.6%) concordant reactive ELISA results were not confirmed. One case was a healthy individual with an equivocal result for both the C6 ELISA and the Serion IgM ELISA, and the other case was a treated Lyme neuroborreliosis patient with a positive result for both the C6 ELISA and the Serion IgM ELISA (Fig. 2).

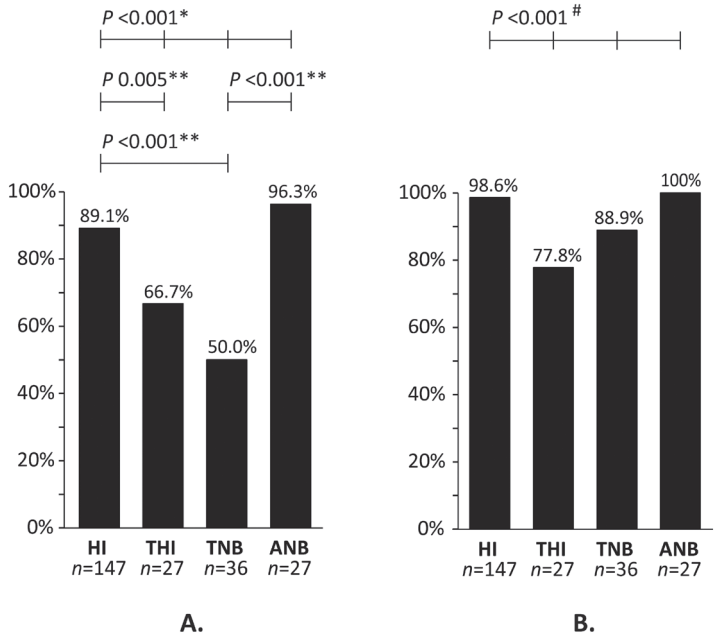

Fig. 3. The concordance between the results of the C6 ELISA and the results of the Serion ELISA (A) and the concordance between the results of the C6 ELISA plus immunoblot strategy and the Serion ELISA plus immunoblot strategy (B). HI, healthy individuals; THI, treated healthy individuals; TNB, treated Lyme neuroborreliosis patients; ANB, active Lyme neuroborreliosis patients; *n*, number of study subjects. Only statistical significant differences are displayed. For all two-group comparisons with a significant difference, the Bonferroni correction was applied (*P*: 0.050/6 = 0.008). */#: Significant difference between the ELISAs (*) and the test strategies (#) in the overall dataset; **: Significant difference between the two-group comparisons of the ELISAs.

Analysis of the 44 discordant ELISA results showed that most cases were Serion ELISA reactive/C6 ELISA negative (*n* = 24; 54.5%) (Fig. 2). Those cases were based on reactivity for the Serion IgM ELISA alone (*n* = 16/24; 66.6%), for the Serion IgG ELISA alone (3/16; 18.8%) or for both the Serion IgM and the Serion IgG ELISAs (5/16; 31.3%). In total, 13/44 (29.5%) discordant ELISA results were confirmed, of which only two were based on C6 ELISA reactive/Serion ELISA negative results. Interestingly, none of the 15 C6 ELISA reactive/Serion ELISA negative results showed a VlsE band. Immunoblot confirmation of the two cases was based on the presence of IgM against OspC (*n* = 2) and p41 flagellin (*n* = 1) (data not shown). In total, 10/24 (41.7%) Serion ELISA reactive/C6 ELISA negative results were confirmed. Five (31.3%) out of the 16 solitary IgM reactive cases were confirmed based on IgM against OspC (*n* = 5) and p41 flagellin (*n* = 1); one also had IgG against OspC and p41 flagellin. One (33.3%) out of the three solitary IgG cases was confirmed based on IgG against p41 flagellin and p18, and 4/5 (80.0%) IgM and IgG reactive cases were confirmed. Two cases had IgM against OspC and p41 flagellin, of whom the second case also had IgG against VlsE and p18, the third case had IgG against OspC and p41 flagellin, and the fourth case had IgG against VlsE and p39 (data not shown). The remaining confirmed case was an active neuroborreliosis patient who had reactive - but discordant - ELISA results. Immunoblot analysis showed IgG against VlsE, p41 flagellin and p39 (data not shown).

CONCORDANCE OF TEST STRATEGY RESULTS

For all groups, the concordance between the C6/IB strategy and the SE/IB strategy was higher than the concordance found between both ELISAs. Only for treated healthy individuals was the concordance still significantly lower than for healthy individuals (21/27 (77.8%) and 145/147 (98.6%), respectively) (*P* < 0.001) (Fig. 3b). Interestingly, no correlation was found between

the C6 ELISA reactive/Serion ELISA negative results and the immunoblot results (r_s = 0.154; *P* = 0.584), but a correlation was found between the Serion ELISA reactive/C6 ELISA negative results and the immunoblot results (r_s = 0.555; *P* = 0.005). Thus, reactive Serion ELISA results were more often confirmed by immunoblot analysis than the reactive C6 ELISA results.

POSSIBLE EXPLANATIONS FOR THE DISCORDANT ELISA AND TEST STRATEGY RESULTS

Discordant ELISA results are caused by variability in the amount and type of antibodies, which, in turn, may be influenced by antibiotic treatment and/or the natural course of clearance of infection. The natural course of clearance of infection may be influenced by the age, sex or recovery of the patient, whether or not a tick bite or EM were observed, the time between end of antibiotic treatment and blood sampling and duration of symptoms before the start of antibiotic therapy. We determined the possible contribution of these factors to the discordance of test results. Analysis of all study participants showed that only antibiotic treatment was strongly associated with discordant ELISA and discordant test strategy results [odds ratio (OR): 10.52; [*P* < 0.001 and OR: 9.98; *P* = 0.014, respectively] (Table 2). Among healthy individuals and Lyme neuroborreliosis patients who were both treated, the time between the end of antibiotic treatment and blood sampling did not contribute to an increased discordance (data not shown). However, solely among treated Lyme neuroborreliosis patients, the C6 ELISA index scores did decrease with increasing time between the end of antibiotic treatment and blood sampling (r_s = −0.408; *P* = 0.013). For the Serion IgM and Serion IgG ELISAs, and among treated healthy individuals, we did not find such a correlation (data not shown). When both treated groups were analyzed separately, older age was associated with a slight increase in the percentage of discordant ELISA results among treated Lyme neuroborreliosis patients (OR: 1.10; *P* = 0.015). In contrast, older age was associated with a lower percentage of discordant test strategy results among treated Lyme individuals, although only slightly (OR: 0.82; *P* = 0.033). There was no association between the recovery status or the duration of symptoms and the percentage of discordant ELISA or test strategy results among treated Lyme neuroborreliosis patients (data not shown).

Table 2. Logistic regression model assessing risk factors for discordant test results among study participants

Test	Parameter	B	SE	Wald	df	*P* value	Odds ratio	95% CI for OR	
								Lower	Upper
ELISAs	Sex (male)	−0.15	0.37	0.17	1	0.684	0.86	0.42	1.78
	Tick bite (yes)	−0.49	0.42	1.38	1	0.240	0.61	0.27	1.39
	EM (yes)	−0.65	0.50	1.70	1	0.193	0.52	0.19	1.39
	Antibiotic treatment (yes)	2.35	0.49	23.49	1	<0.001[a]	10.52	4.06	27.26
	Age	0.01	0.01	0.21	1	0.647	1.01	0.98	1.03
Test strategies	Sex (male)	−0.47	0.64	0.54	1	0.462	0.62	0.18	2.20
	Tick bite (yes)	−0.83	0.74	1.26	1	0.262	0.44	0.10	1.86
	EM (yes)	0.78	0.72	1.15	1	0.283	2.17	0.53	8.97
	Antibiotic treatment (yes)	2.30	0.93	6.10	1	0.014[a]	9.98	1.61	61.92
	Age	−0.02	0.02	0.94	1	0.333	0.98	0.93	1.02

The logistic regression model gives the probability that a discordant test result will be found (*n*, 226; 11 active Lyme neuroborreliosis patients did not fill out the Lyme-specific questionnaire and were, thus, excluded from the calculations). B, coefficient for the constant; SE, standard error; df, degrees of freedom for the Wald Chi-square test; CI, confidence interval; OR, odds ratio

a. Statistically significant.

DISCUSSION

In this study, we compared two standard two-tier test strategies by using two screening ELISAs and a conformational immunoblot on various well-defined Dutch Lyme patients and healthy individuals. High concordances between the results of the test strategies were found for healthy individuals and active Lyme neuroborreliosis patients groups (range 98.6-100%); however, low concordances were observed for Lyme neuroborreliosis patients and healthy individuals who had been treated for Lyme borreliosis in the past (range 77.8-88.9%). Discordant test results represent variability in the amount and type of antibodies, which, in turn, may be influenced by antibiotic treatment and/or the natural course of clearance of infection. Of the investigated factors affecting the natural clearance of the infection, only age contributed to discordant ELISA or test strategy results within both treated groups, but only to a minimal extent. Older age was associated with an increase of discordant ELISA results among treated Lyme neuroborreliosis patients and with a decrease of discordant test strategy results among treated healthy individuals. The significantly higher discordance between the ELISA results in the two treated groups compared to the almost concordant ELISA results in active Lyme neuroborreliosis patients (96.3%) and healthy individuals (89.1%) was associated to antibiotic treatment against Lyme borreliosis. This suggests that antibiotic treatment influences the pace at which the detected serum antibodies wane.

Furthermore, this study showed that the Serion ELISA had more positive results than the C6 ELISA and also led to a higher percentage of immunoblot-confirmed results. This finding implies that serum antibodies against the C6 peptide wane faster than other *Borrelia*-specific serum antibodies. In this study, we found a significant correlation among treated Lyme neuroborreliosis patients between the decrease in C6 ELISA index scores and a longer time between the end of antibiotic treatment and blood sampling. Although the number of cases in our study is small, other studies also showed a decline of C6 antibodies and a >4-fold decrease in C6 ELISA results within one year after antibiotic treatment [20, 21]. In 9/13 (69.2%) immunoblot-confirmed sera, for which discordant ELISA results were found, the presence of IgM against OspC, and in some cases p41 flagellin, was found. In 10/13 cases (76.9%), confirmation was based on a reactive Serion ELISA result only. Since both OspC and p41 flagellin are part of the whole-cell lysate used in the Serion ELISA, this might explain the higher positive rate for this ELISA. Aguero-Rosenfeld et al. [22] also reported the persistence of IgM against OspC and p41 flagellin in more than half of the patients in their study during a follow-up at one year. The persistence of the immune response against OspC and p41 flagellin up to 20 years after successful treatment has also been described by Kalish et al. [4]. To emphasize, the presence of *Borrelia*-specific serum antibodies is no proof of an active Lyme borreliosis infection or proof of a false-positive reaction of the ELISA used, but could well be explained by a previous, cleared Lyme borreliosis infection, with or without antibiotic treatment.

Surprisingly, all 15 reactive C6 ELISA results with negative Serion ELISA results failed to show a VlsE band in the immunoblot analysis. A C6 ELISA positivity could be expected, since 10/15 (66.7%) cases were treated for Lyme borreliosis in the past, but the absence of a VlsE band for all cases was unexpected. It could be explained by a reduced sensitivity of the immunoblot [23] or by a difference in the accessibility of the C6 peptide on the immunoblot. The C6 peptide is not exposed on the surface of VlsE and becomes available only when VlsE is processed after a *Borrelia* infection has occurred and it is, therefore, suggested that C6 elicits a different antibody response compared to VlsE, which is supported by this study [12, 15].

Although this study underlines the importance of the two-tier test strategy, the analysis of the third strategy, SE + C6/IB, was also of interest, as this strategy resulted in the highest number of sera for which *Borrelia*-specific serum antibodies were detected. A disadvantage was the high

number of immunoblot tests that were needed to get the final results. The strategy could be simplified by not confirming concordant positive ELISA results with immunoblot. This simplified SE + C6/IB would substantially decrease the number of immunoblots needed from 99 to 45 (−54.5%), which is also less compared to the C6/IB strategy (45 vs. 75 (−40.0%)) or the SE/IB strategy alone (45 vs. 84 (−46.4%)). The final results would almost be similar, as only one treated neuroborreliosis patient would be recorded differently (both ELISAs positive/immunoblot negative). Since the immunoblot is more labor-intensive, expensive and the interpretation more subjective compared to an ELISA, this simplified SE + C6/IB strategy is interesting, as other studies also underline the potential of a two-ELISA strategy for the diagnosis of Lyme borreliosis [24, 25].

The selection criteria used in our study ruled out the inclusion of other than Lyme neuroborreliosis patients. Since Lyme neuroborreliosis is the most common clinical presentation of disseminated Lyme borreliosis in the Netherlands and the criteria for Lyme neuroborreliosis, as proposed by the EFNS [16], are clear and easy to apply, this ensured the inclusion of true Lyme neuroborreliosis cases. A potential bias could be the lower age of the healthy individuals compared to the other three groups. Furthermore, eight patients were included twice in this study, both as an active Lyme neuroborreliosis patient and later as a treated Lyme neuroborreliosis patient. Nevertheless, we argue that the influence of these potential biases are limited, since they most likely do not affect the performance of the ELISAs and the immunoblot, or, if so, the possible effect should be equal for all tests concerned. Finally, the number of study participants in our study is limited and future studies including more patients and healthy controls, as well as different Lyme manifestations, are needed to strengthen our findings. Future studies could also investigate the effect of the host immune response and the infecting *Borrelia* species on test results.

To conclude, this study showed a lower test agreement in healthy individuals and Lyme neuroborreliosis patients who were both treated for Lyme borreliosis in the past. We showed that antibiotic treatment influences serological test results and that the effect differs for the different assays. Although the number of cases is limited and we did not take into account the possible influence of the different clinical manifestations, we do believe that antibiotics contribute to the variation in the kinetics of the antibodies directed against the different *Borrelia* antigens used in the tests. Although only two commercial ELISAs and one commercial immunoblot were tested, our data suggest that this holds true for other assays based on different antigens and/or different *Borrelia* strains. This study emphasizes that care should be taken when Lyme serology is considered and symptoms are non-specific. It supports the general opinion that serological testing for *Borrelia*-specific serum antibodies should only be performed in case of a high a priori chance of Lyme borreliosis. To our knowledge, this is the first study that gives an in-depth insight into the diagnostic challenges which arise when individuals have been treated for Lyme borreliosis in the past.

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COMPLIANCE WITH ETHICAL STANDARDS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The regional Medical Research Ethics Committees United (Nieuwegein) approved this study (MEC-U: NL36407.100.11).

INFORMED CONSENT

Informed consent was obtained from all study participants.

OPEN ACCESS

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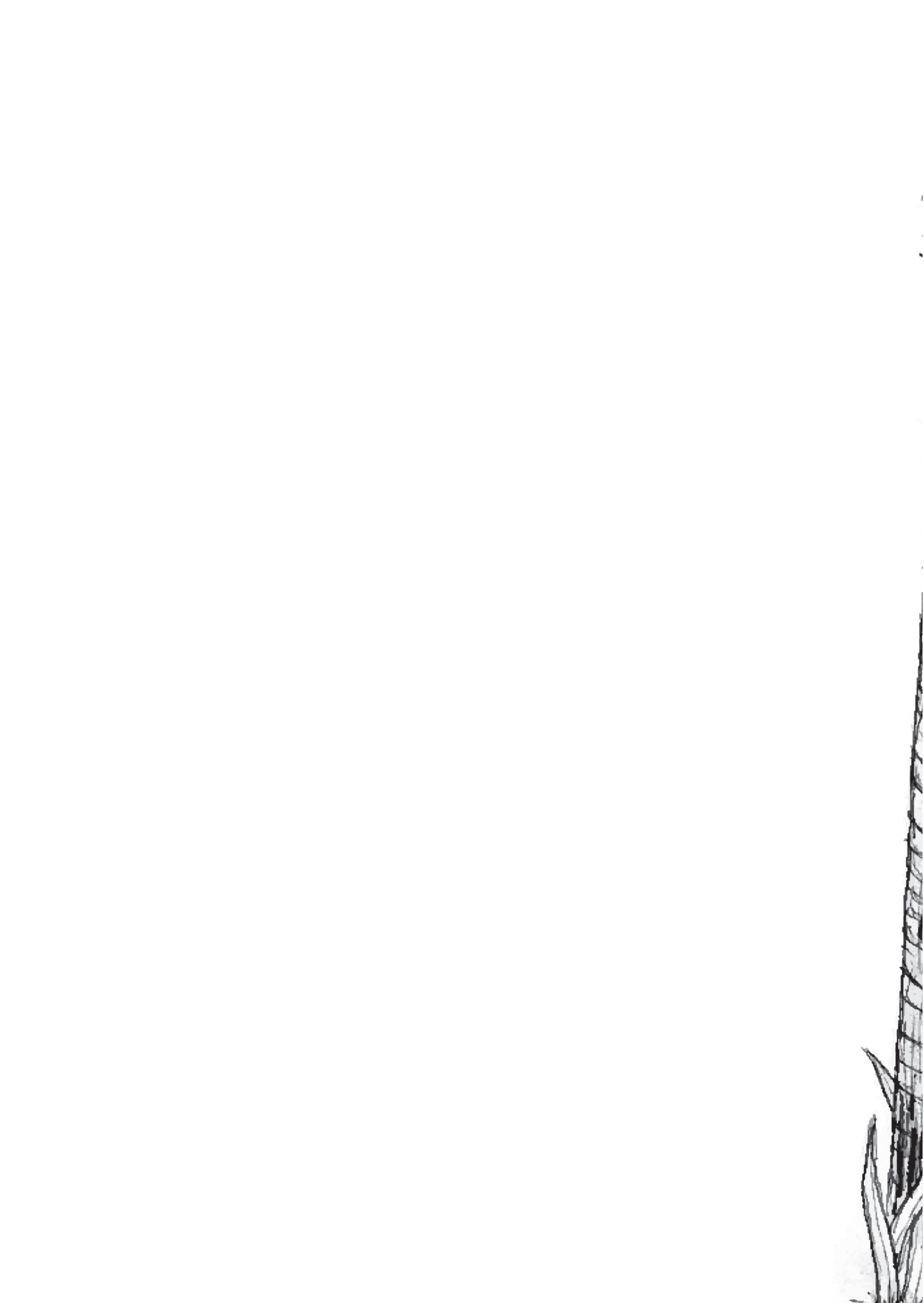

8 GENERAL DISCUSSION

INTRODUCTION

Lyme borreliosis (LB) is the most commonly reported tick-borne infection in countries with moderate climates in Europe, North America and Asia [1]. It is a multisystem disease that can cause local, early disseminated and late disseminated infection, and can involve different organs and/or body parts such as skin, nervous system, heart, joints and eyes [1]. Typical erythema migrans (EM) lesions can be clinically diagnosed and the diagnosis of other Lyme manifestations requires laboratory confirmation [1]. The diagnosis of LB, however, can be challenging since clinical symptoms are often non-pathognomonic and a gold-standard test is lacking. The most recommended diagnostic tool for LB diagnostics is based on the detection of *Borrelia*-specific antibodies [2], but this method is hampered by several shortcomings, such as a low sensitivity in the early phase of the infection due to the kinetics of the antibody response, false-positive test results due to cross-reactivity, and the persistence of *Borrelia*-specific antibodies after a cleared infection [3]. Also, many different antibody assays for the diagnosis of LB are on the market. The performance characteristics of many of these assays have been studied, but results are often difficult to interpret due to variability in study set-up, heterogeneity among patient populations, and poor reporting of study characteristics [4]. Ideally, diagnostic accuracy studies should be prospectively planned using a cross-sectional study design in a clinical setting, where the test will be used in practice [4]. Yet, the number of published reports that evaluated tests to be used for LB diagnostics by means of a cross-sectional study design was much lower than the number of published reports that evaluated tests used for LB diagnostics by means of a case-control study design (n = 18 and n = 57, respectively) [4]. In general, a case-control study design is easier to perform, but has a risk of introducing bias as such a study excludes patients who are difficult to diagnose.

In our experience, medical specialists are frustrated with the lack of adequate diagnostic tools that enable them to confirm or reject with certainty the profound and rightful question of the patient 'Do I suffer from LB?'. For appropriate LB diagnosis, tools are needed with high sensitivity and specificity, which can discriminate between active disease and a previous, yet cleared, infection. Triggered by these needs, the work in this thesis focused on research into LB diagnostics to gain more insight and improve LB diagnostics, whereby the emphasis was laid on indirect detection methods investigating the humoral and cellular immune response against an infection with *Borrelia burgdorferi* sensu lato. The humoral immune response was investigated as the detection of *Borrelia*-specific antibodies is the recommended and most widely used diagnostic tool for LB diagnosis. The cellular immune response was investigated as it was hypothesized that measuring a patients' cellular immune response against an infection with *B. burgdorferi* s.l. may be of added value for those cases in which the humoral immune response falls short [5]. A lot of effort was put into setting up the various study populations that were used for the studies in this thesis, for which well-defined patients and controls were used. As clear case definitions are put down for European Lyme neuroborreliosis (LNB) [6], active LNB served as a proxy for active disease. Consequently, most of the lessons learned from the research conducted in this thesis are only applicable for patients suspected of LNB and do not necessarily translate to other Lyme manifestations.

COMBINING THE HUMORAL AND CELLULAR IMMUNE RESPONSE

A DIAGNOSTIC ALGORITHM FOR LNB DIAGNOSTICS SHOWS PROMISING RESULTS

The most important finding of the research conducted in this thesis are the promising results described in **chapter 6**, as these will aid clinicians in diagnosing LNB. Therefore, this chapter will be discussed first, subsequently followed by those covering the humoral immune response (**chapters 5** and **7**, respectively), and those covering the cellular immune response (**chapters 4**, **3** and **2**, respectively).

In **chapter 6**, a cross-sectional study design was chosen to evaluate seven commercial antibody assays for LNB diagnostics. This cross-sectional study design comprised all consecutive patients from whom a cerebrospinal fluid (CSF) and serum sample was drawn in the routine clinical setting of our hospital during a certain timeframe, who were retrospectively included. **Chapter 6** also included seven multiparameter analyses, one for each antibody assay under evaluation. These multiparameter analyses showed that for most antibody assays, the sensitivity to diagnose LNB could be improved by including other parameters.

The multiparameter analyses in **chapter 6** also provided insight into the relative contribution of the included parameters for LNB diagnostics and, thus, which of those may be useful in a diagnostic algorithm. Of the 13 included parameters, the following parameters were most predictive for LNB: (i) the presence of intrathecally produced *Borrelia*-specific antibodies, preferably determined according to Reiber and Peter [7], (ii) the presence of *Borrelia*-specific serum antibodies (i.e., determined using a two-tier test strategy), (iii) an elevated level of the B-cell chemokine (C-X-C motif) ligand 13 (CXCL13) in the CSF, (iv) a Reibergram classification to area 3 (i.e., a dysfunctional blood-CSF barrier together with the presence of intrathecally produced total antibodies (immunoglobulin [Ig]M and/or IgG)), and (v) an elevated CSF-leucocyte count (i.e., ≥5 leucocytes/µl [pleocytosis]). Although various studies also reported the additional value of these parameters for LNB diagnostics [6, 8-10], our study is the first to show the relative importance of these parameters and, thus, the first to show which of those are most important and may be used in a diagnostic algorithm for LNB diagnostics. The results of our study also showed that both the humoral and the cellular immune response against an infection with *B. burgdorferi* s.l. are important for the diagnosis of LNB. Furthermore, the results showed that individual parameters can either be positive, or negative, and still be of added value at large.

Table 1 shows an example of the results of the multiparameter analysis for one of the antibody assays under investigation (i.e., the Serion enzyme-linked immunosorbent assay [ELISA]), which is currently in use at the Diakonessenhuis Hospital, Utrecht, the Netherlands. In **chapter 6**, patients were classified as either definite LNB, possible LNB or non-LNB patient using the EFNS guidelines and a consensus strategy for the presence of intrathecally produced *Borrelia*-specific antibodies. This consensus strategy entailed that the majority of the antibody assays tested on CSF-serum pairs in this study had to show a pathological *Borrelia*-specific (IgM and/or IgG) antibody index (AI) value to prove intrathecal *Borrelia*-specific antibody synthesis. Consequently, the classification of patients based on the IgM and IgG test results of a single antibody assay could differ from those obtained using the consensus strategy. However, the classificition of patients using the IgM and IgG test results of the Serion ELISA did not differ from the classificition of patients using the concensus strategy. The sensitivity of the Serion ELISA to diagnose definite and possible LNB patients was 75.0%, and this percentage increased to 100% using multiparameter analysis. The specificity of the Serion ELISA was 95.7%, and this slightly decreased to 94.2% using multiparameter analysis. Based on the results of the multiparameter analysis of the Serion ELISA, all definite and possible LNB patients including a limited number of non-LNB patients were predicted to have LNB (Table 1).

Interestingly, for some of the possible LNB and non-LNB patients who were predicted to have LNB based on the multiparameter analysis of the Serion ELISA, the results of some of the parameters included in this analysis were comparable (Table 1). Consequently, further investigation is needed to investigate if other parameters can be found that can distinguish these patients. As expected, the number of parameters that was positive among definite LNB patients was higher than the number of positive parameters among possible LNB patients, which may be caused by the duration of disease, as is discussed in various sections hereafter. Due to the cross-sectional study design and the low annual incidence rate of LNB in the Netherlands (i.e., 2.6 per 100,000

in 2010 [11]), the number of definite and possible LNB patients in this chapter was limited. The results should therefore be confirmed in a prospective, multicenter study aiming at including more definite and possible LNB patients as well as controls with other proven non-LNB diseases.

Table 1. Diagnostic algoritm for the Serion ELISA using multiparameter analysis among 154 (98.7%) of the 156 study participants (i.e., for two patients, one definite Lyme neuroborreliose [dLNB] and one non-LNB patient, the Serion ELISA result was missing due to insufficient sample material). The 13 parameters included in the multiparameter analysis are shown in separate columns and sorted by their relative importance in predicting LNB from left (highest) to right (lowest). In total, 24 study participants were predicted to have LNB (panel A), including all (*n* = 16) dLNB and possible (p) LNB patients as well as 8 non-LNB patients. One-hundred thirty study participants were predicted not to have LNB (panel B), and these were all non-LNB patients. This resulted in a sensitivity of 100% (16/16) for predicting LNB, and a specificity of 94.2% (130/138) for predicting non-LNB. Black boxes indicate a positive and white boxes indicate a negative test result for the respective parameter.

	The 13 test parameters used in the multiparameter analysis														
	Serion ELISA[a]	CSF-CXCL13[b]	Two-tier serology[c]	Pleocytosis[d]	Reibergram[e]; area 3	Reibergram[e]; all areas separately	Reibergram[e]; area 1	Reibergram[e]; area 2+3	Reibergram[e]; area 3+4	Reibergram[e]; area 4	Reibergram[e]; area 2	*Borrelia* PCR on CSF	Total protein in CSF	Classification of patients[f]	No. of patients
Relative importance[g]	250	191	178	137	118	101	92	72	64	52	37	33	18		
A	■	■	■	■	■	area 3		■	■				■	dLNB	1
	■	■	■	■	■	area 3		■	■			■	■	dLNB	1
	■	■	■	■	■	area 3		■	■			■	■	dLNB	1
	■	■	■	■	■	area 3		■	■				■	dLNB	1
	■	■	■	■	■	area 3		■	■					dLNB	1
	■	■	■	■	■	area 3		■	■				■	dLNB	1
	■	■	■	■		area 1	■							dLNB	1
	■	■	■	■		area 2		■			■			dLNB	1
	■		■			area 2		■			■			pLNB	1
	■		■			area 4			■	■				pLNB	2
	■			■		area 2		■			■			dLNB	1
	■					area 4			■	■				non-LNB	1
		■		■	■	area 3		■	■				■	non-LNB	1
		■		■	■	area 3		■	■					pLNB	1
			■	■		area 1	■							pLNB	2
			■	■		area 1	■							non-LNB	2
			■	■		area 2		■			■			pLNB	1
			■			area 2		■			■			non-LNB	2
			■			area 2		■			■		■	non-LNB	1
			■			area 4			■	■				non-LNB	1
														Total:	24
B	■		■			area 1	■						■	non-LNB	1
	■		■			area 1	■							non-LNB	2
	■					area 1	■							non-LNB	2
			■			area 1	■							non-LNB	22
				■		area 1	■							non-LNB	8
				■		area 2		■			■		■	non-LNB	3
				■		area 2		■			■			non-LNB	2

Table 1. Continued.

	The 13 test parameters used in the multiparameter analysis														
	Serion ELISA[a]	CSF-CXCL13[b]	Two-tier serology[c]	Pleocytosis[d]	Reibergram[e]; area 3	Reibergram[e]; all areas separately	Reibergram[e]; area 1	Reibergram[e]; area 2+3	Reibergram[e]; area 3+4	Reibergram[e]; area 4	Reibergram[e]; area 2	*Borrelia* PCR on CSF	Total protein in CSF	Classification of patients[f]	No. of patients
Relative importance[g]	250	191	178	137	118	101	92	72	64	52	37	33	18		
				■		area 4			■	■				non-LNB	5
						area 1	■							non-LNB	67
						area 2		■			■			non-LNB	9
						area 2		■			■		■	non-LNB	3
						area 4			■	■				non-LNB	6
														Total:	130

a. The results of the Serion ELISA were based on the combined (IgM and IgG) antibody index (AI) results.
b. The final B-cell chemokine (C-X-C motif) ligand 1 (CXCL13) result in the cerebrospinal fluid (CSF) was based on the combined results of the two CXCL13 assays evaluated in **chapter 4**, and was considered negative when either one, or both assays were negative, and positive when both assays were positive.
c. Two-tier serology on serum was performed using the C6 ELISA as a screenings test, and positive (and equivocal) C6 ELISA results were confirmed using the *recom*Line IgM and IgG immunoblot (IB).
d. Pleocytosis is based on a CSF cell count ≥5 leucocytes/µl.
e. The Reibergram areas are defined as described by Reiber [12]. Reibergram area 1: a normal blood-CSF barrier without intrathecal total IgM and/or IgG synthesis >10%, Reibergram area 2: a dysfunctional blood-CSF barrier without intrathecal total IgM and/or IgG synthesis >10%, Reibergram area 3: a dysfunctional blood-CSF barrier with intrathecal total IgM and/or IgG synthesis >10%, Reibergram area 4: intrathecal total IgM and/or IgG synthesis >10% with a normal blood-CSF barrier, Reibergram area 2+3: a dysfunctional blood-CSF barrier irrespective of intrathecal total IgM and/or IgG synthesis >10%, Reibergram area 3+4: intrathecal total IgM and/or IgG synthesis >10% irrespective of the functionality of the blood-CSF barrier.
f. The classification of patients was done based on the criteria of the European Federation of Neurological Societies (EFNS [6]) and consensus strategy and comprised the presence of clinical symptoms suggestive of LNB without another cause, pleocytosis and intrathecal *Borrelia*-specific antibody synthesis. The consensus strategy entailed that intrathecal *Borrelia*-specific antibody synthesis was only considered proven if the majority of the CSF-serum assays under investigation (see **chapter 6**) showed a pathological *Borrelia*-specific IgM and/or IgG AI value (≥1.5).
g. The relative importance of each predictor variable was calculated as described by Liaw and Wiener [13].

THE HUMORAL IMMUNE RESPONSE

THE EDGE EFFECT IN AN ELISA PLATE CAN INFLUENCE ANTIBODY TEST RESULTS

The multiparameter analyses performed in **chapter 6** showed that the humoral immune response is important in LNB diagnostics. Of the 13 parameters that were investigated in this chapter, the two most important ones were the detection of intrathecally produced *Borrelia*-specific antibodies and the detection of *Borrelia*-specific serum antibodies, with a mean rank of 1.7 and 2.4, respectively. In general, the detection of pathogen-specific antibodies is often done using an ELISA [3]. ELISAs, however, can have various shortcomings that have to be considered. One of these shortcomings is the presence of a so-called edge effect. The edge effect is a phenomenon in which the outer wells of a 96-well ELISA plate can have higher optical density (OD) values than the OD values of the inner wells. This this may be caused by temperature differences across the plate [14-16], or by differences in surface properties or unequal protein binding [16-18]. If an ELISA suffers from an edge effect, a patient sample can have a negative test result when tested in an inner well, and a positive test result when tested in an outer well. Well-to-well variations in an ELISA plate might, thus, influence the measurements of the antibody concentration in these wells. This has an effect on both the intra- as well as the interassay variation [19, 20], which negatively influences the reliability of the assay and can complicate the interpretation of test results.

For the detection of intrathecally produced *Borrelia*-specific antibodies, the presence of an edge effect may, thus, have an impact, as the CSF and serum of a patient presumed to have LNB, and which are used to calculate a CSF/serum quotient, must be tested simultaneously in different wells of the same ELISA plate. As was shown in **chapter 5**, the commercial Enzygnost Lyme link variable major protein-like sequence, expressed (VlsE)/IgG ELISA, which was one of the antibody assays that was evaluated in **chapter 6**, indeed suffers from an edge effect. By simulation, of the 149 study participants who were all part of the slightly larger study population used in **chapter 6** (and **4**), and from whom CSF and serum was tested using the Enzygnost Lyme link VlsE/IgG ELISA, the edge effect could have resulted in 15 (10.1%) false-positive and two (1.3%) false-negative *Borrelia*-specific IgG AI results. It was also shown that by a slight adjustment in the standard protocol of the manufacturer, using only wells with a similar location (i.e., outer wells only, or inner wells only), these erroneous results can be prevented. As the detection of pathogen-specific antibodies plays an important role in the diagnosis of many infectious diseases, these results underline the importance of a thorough validation of such assays before use in routine diagnostics as is also required, e.g., by the ISO 15189 accreditation [21].

Besides variations in the measurement of antibody concentrations using the same antibody assay due to the presence of an edge effect, different antibody assays can also vary in sensitivity [4, 22], as was shown in **chapter 6**. Differences in sensitivity between antibody assays are most likely caused by variations in the antigen composition of these assays. Antigens in the antibody assay may not always match the antigens expressed by the *Borrelia burgdorferi* s.l. strain causing disease, and this can be caused by the intra- and interspecies heterogeneity of *B. burgdorferi* s.l. [9, 23-27] and/or the antigenic variation that the bacterium can apply during the course of disease [28].

The difference in sensitivity between various antibody assays can also be caused by antibiotic treatment prior to blood sampling, as antibiotics have been shown to impede or reduce the activated immune response [29-33]. These effects seem to be antibody-specific, as C6 antibodies appear to wane faster than antibodies against whole-cell lysates [34, 35] or p39 [34].

ANTIBIOTIC TREATMENT INFLUENCES THE ANTIBODY RESPONSE

In **chapter 7** it was shown that antibiotic treatment has an effect on the antibody response. A high degree of concordance was found between the test results of the two screening ELISAs (i.e., the C6 and the Serion ELISA) among untreated LNB patients and untreated healthy individuals. Similarly, a high degree of concordance was found between the test results of the confirmation test (i.e., the *recom*Line immunoblot) performed on equivocal and positive test results of both screening ELISAs among untreated LNB patients and untreated healthy individuals. In contrast, less concordance was seen between these test results among treated LNB patients and treated healthy individuals and this lower concordance was shown to be associated with antibiotic treatment. Discordant test results were explained by the faster waning of antibodies directed against the C6 peptide than those directed against whole-cell lysates of *B. burgdorferi* s.l.. These results are in line with those that have been reported by others [34, 35]. Interestingly, Peltomaa et al. [34] suggested that a decline in C6 antibody titers after antibiotic treatment might be associated with a shorter duration of disease, and warrants further investigation. Most of the discordant test results in our study were linked to the presence of IgM against outer surface protein (Osp)C, and for some cases also against p41 flagellin, as published previously [36, 37]. Overall, the results from this study as well as those in **chapter 5** underline the difficulties surrounding the interpretation of antibody test results, and these difficulties should be taken into consideration when serology is performed.

THE CELLULAR IMMUNE RESPONSE

THE POTENTIAL OF CSF-CXCL13 FOR LNB DIAGNOSTICS

The multiparameter analyses in **chapter 6** also showed that the cellular immune response, by measuring CXCL13 levels in the CSF, is important in LNB diagnostics. Of the 13 parameters investigated in this chapter, determining the CSF-CXCL13 level was ranked third, with a mean rank of 2.6. This rank was just slightly higher than the mean rank of 2.4 found using two-tier serology on serum. In the last two decades, many studies have reported elevated levels of CXCL13 in the CSF of patients with early LNB compared to other neurological diseases, including Guillain Barré syndrome and Bell's palsy [38-40]. One of the difficulties, however, is the large range of cutoff values that have been reported, ranging from 18 pg/ml to 1,229 pg/ml [41-47], which might be caused by a number of reasons, such as differences in methodology, the use of different platforms or the use of different calculation methods [41]. Yet, different cutoff values were also found using the same assay [42-47]. This suggests that other mechanisms are involved as well, such as differences in the study set-up and, consequently, the groups used to determine the cutoff levels. Differences in sample handling [10], the inter- and intraspecies heterogeneity of *B. burgdorferi* s.l. and/or host genetic factors [48, 49] may also play a role. The CSF-CXCL13 results used in **chapter 6** were based on the combined results of the two CXCL13 assays (i.e., the Quantikine CXCL13 ELISA and the *recom*Bead CXCL13 assay) that were evaluated in **chapter 4**. In this chapter, both CXCL13 assays showed different cutoff values, and these cutoff values also differed from those found in the literature using the same assays [41, 42]. Therefore, further investigation is warranted to elucidate the causes of these differences and raises the question of whether an international reference standard for CXCL13 in the CSF could be established.

THE POTENTIAL OF CSF-CXCL13 AMONG POSSIBLE LNB PATIENTS

The determination of CSF-CXCL13 levels seems especially useful among possible LNB patients. The production of CXCL13 in the CSF preceded the humoral immune response in three of the eight possible LNB patients in **chapter 4**, all of whom had pleocytosis. This is in line with the suggestion made by some that possible LNB patients with pleocytosis represent early LNB cases for whom the antibody response is building up, but is still too low to be detected at the moment of sampling CSF and/or blood, and that possible LNB patients with intrathecal *Borrelia*-specific antibody synthesis most likely have had a previous LNB, or another disease [42, 50]. The diagnosis of early LNB for these three patients was supported by the Reibergram analyses showing a dysfunctional blood-CSF barrier and intrathecal total-IgM synthesis in the absence of intrathecal total-IgG synthesis [8, 51]. For the three possible LNB patients with pleocytosis and a positive CSF-CXCL13 result, and for whom intrathecally produced *Borrelia*-specific antibodies were not detected using the IDEIA LNB assay, the presence of these antibodies was shown by at least two other antibody assays evaluated on CSF-serum pairs in **chapter 6**. This suggests that the sensitivity of the IDEIA LNB assay used in the current study is lower in the early stages of LNB, as was reported before [52, 53]. The diagnosis of early LNB for these three possible LNB patients was also supported by the results of the multiparameter analysis of the Serion ELISA (Table 1) and the results of the six other multiparameter analyses in **chapter 6** (individual data not shown), as these patients were all predicted to have LNB.

THE ABSENCE OF ELEVATED CSF-CXCL13 LEVELS AMONG POSSIBLE LNB PATIENTS

Negative CSF-CXCL13 results were found for five (37.5%) of the eight possible LNB patients in **chapter 4**. Four of them had pleocytosis in the absence of intrathecally produced *Borrelia*-specific antibodies, and even though CSF-CXCL13 results were negative for these four patients, we believe that they represent early LNB patients based on the rationale mentioned in the previous paragraph. Interestingly, all suffered from cranial neuropathy of either the seventh (n = 3) or sixth (n = 1) nerve. One of them (nerve VII paresis) had a dysfunctional blood-CSF barrier and

CSF-CXCL13 levels were elevated in both assays (53 pg/ml using the CXCL13 ELISA and 103 pg/ml using the *recom*Bead CXCL13 assay); however, these levels were below the assay-specific cutoff value. This patient had noticed a tick bite approximately 3 weeks before the LP, and symptoms had started 5 days prior to the LP and the facial palsy for this patient resolved with antibiotic therapy. Two other patients (nerve VII paresis, both) reported a short duration of symptoms (8 and 13 days, respectively), and one of them had a dysfunctional blood-CSF barrier. The other patient had intrathecal *Borrelia*-specific IgM synthesis detected using one of the other antibody assays (i.e., the Enzygnost ELISA) evaluated on CSF-serum pairs (**chapter 6**). For both patients, the facial palsy resolved with antibiotic therapy. The fourth possible LNB patient (nerve VI paresis) had symptoms for 39 days, and these symptoms also resolved with antibiotic therapy. The diagnosis of early LNB for these four possible LNB patients is further supported by the results of the multiparameter analysis of the Serion ELISA (Table 1) as well as by the results of most of the other multiparameter analyses in **chapter 6** (individual data not shown). One possible LNB patient (i.e., the one with nerve VII paresis, a tick bite, and symptom duration of 5 days, who responded well to antibiotic treatment for LNB) was predicted not to have LNB in the multiparameter analysis of the IDEIA LNB assay and the *recom*Line immunoblot. Another possible LNB patient (i.e., the one with nerve VI paresis and a symptom duration of 39 days, which resolved with antibiotic therapy for LNB) was predicted not to have LNB in the multiparameter analysis of the C6 ELISA.

The fifth possible LNB patient had intrathecal *Borrelia*-specific antibody synthesis detected using the IDEIA LNB assay in the absence of pleocytosis (**chapter 4**). This patient suffered from peripheral neuropathy and had symptoms for almost 300 days. The absence of a positive CSF-CXCL13 result for this patient is in line with the hypothesis that possible LNB patients with intrathecal *Borrelia*-specific antibody synthesis most likely have had a previous LNB [42, 50]. In **chapter 6**, this patient was predicted to have LNB in all multiparameter analyses (individual data not shown), except for the analysis that included Serion ELISA (Table 1). Gubanova et al. [54] also reported the absence of a positive CSF-CXCL13 result in a patient suffering from peripheral neuropathy, but this patient had a short duration of symptoms and in the CSF from a second lumbar puncture (LP) almost two months later an elevated CSF-CXCL13 level was found.

As mentioned above, the majority of the possible LNB patients with pleocytosis and a negative CSF-CXCL13 result suffered from cranial neuropathy. This suggests that these patients represent a subgroup of patients who exhibit a different immune response and this is in line with a recent publication of Ogrinc et al. [55]. Consequently, a negative CSF-CXCL13 result, in our opinion, does not rule out (possible) LNB and underscores the plethora of clinical presentations and immune responses in LNB and should be taken into account when interpreting CSF-CXCL13 test results.

Even though CXCL13 is a non-specific marker [45, 46, 56-63], it is more specific than the CSF-leucocyte count, and, dependent on the antibody assay used, more sensitive than the detection of intrathecally produced *Borrelia*-specific antibodies (**chapter 6**). Still, the added value of CXCL13 in CSF should be investigated further as the number of definite LNB and possible LNB patients was limited. This was due to the cross sectional study design that was chosen combined with the low annual incidence rate of LNB in the Netherlands (i.e., 2.6 per 100,000 in 2010 [11]). Like in **chapter 6**, the results in **chapter 4** should be confirmed in a prospective study aiming at including more definite and possible LNB patients as well as controls with other proven non-LNB diseases. Still, other non-specific CSF changes can also aid in the diagnosis of LNB such as elevated protein levels, a dysfunctional blood-CSF barrier, and intrathecal synthesis of total IgM and/or total IgG [8, 51]. Indeed, some of these parameters can be helpful, as an association was seen between elevated CSF-CXCL13 levels, a dysfunctional blood-CSF barrier, and intrathecal total-antibody synthesis, especially among definite LNB patients (**chapters 4** and **6**). Elevated CSF-CXCL13 levels, however, were also seen among definite LNB patients who neither had a dysfunctional blood-

CSF barrier nor intrathecal total-antibody synthesis, and among definite LNB patients who had a dysfunctional blood-CSF barrier without intrathecal total-antibody synthesis (**chapters 4** and **6**). This, again, underlines the potential of a diagnostic algorithm for (optimized) LNB diagnostics.

THE (IM)POSSIBILITIES OF THE IFN-γ ELISPOT ASSAY FOR LNB DIAGNOSTICS

Other tests that focus on the cellular immune response are interferon-gamma (IFN-γ) release assays (IGRAs). These assays can either measure the amount of IFN-γ produced ex vivo by T cells, or count the number of T cells that produce IFN-γ after isolating and subsequently stimulating peripheral blood mononuclear cells (PBMCs) with pathogen-specific antigens [64]. The research in this thesis focused on the latter of these two methods by evaluating two enzyme-linked immunospot (ELISpot) assays. An IFN-γ based assay was chosen as IFN-γ is important in infectious diseases due to its various immunomodulatory effects, both on the innate as well as on the acquired immune response [65, 66]. IFN-γ is a strong activator of macrophages and can enhance the processing and subsequent presentation of antigens in both class I and class II pathways as well as induce the secretion of pro-inflammatory cytokines [65, 66]. IFN-γ can also induce chemotaxis, apoptosis and the production of antimicrobial (and antiviral) mediators, regulate antibody production and enhance opsonization [65, 66]. Consequently, IFN-γ has been studied in various bacterial (*Mycobacteria*, *Salmonella*, *Listeria* or *Staphylococcus*), viral (Varicella Zoster Virus, Hepatitis C virus, cytomegalovirus or severe acute respiratory syndrome coronavirus 2) and parasitic (*Leishmania*) infections [66-71]. IFN-γ has also been used to monitor the effectiveness of vaccines to induce cell-mediated immunity [72], as has been done in case of measles virus [73] and varicella zoster virus [74]. For diagnostic purposes, IFN-γ based assays have mostly been used for tuberculosis (TB) [75]. For LB diagnostics, robust validations of IFN-γ based assays on well-defined study populations are lacking [76-82]. Nonetheless, various laboratories offer these assays for the diagnosis of LB, and patients may even receive prolonged courses of antibiotics assuming that a positive test result for LB is indicative for active disease [82].

THE IFN-γ ELISPOT ASSAY IS NOT USEFUL FOR LNB DIAGNOSTICS

In this thesis, an in-house *Borrelia* ELISpot assay (**chapters 2** and **3**), and the commercial LymeSpot assay (**chapter 3**) were evaluated for LNB diagnostics. The commercial LymeSpot assay is widely used, for example in Germany [76, 77, 79], even though a proper validation is lacking as the manufacturer of this assay did not have access to well-defined patient populations. Therefore, they greeted our effort to validate the LymeSpot assay. Using a case-control design, both active and treated Lyme neuroborreliosis patients (classified according to the EFNS guidelines [6]), as well as treated and untreated healthy individuals were included. The research in both chapters showed that the IFN-γ ELISpot assay cannot be used to diagnose active LNB, irrespective of which assay was used. Both assays showed that the IFN-γ ELISpot reactivity (i.e., the numbers of *Borrelia*-specific IFN-γ producing T cells, or spot forming cells [SFC]) was comparable between active LNB patients, treated LNB patients and treated healthy individuals and that this reactivity was more pronounced than the reactivity seen among untreated healthy individuals. These results were confirmed using receiver operating curve (ROC) analysis, as, irrespective of the IFN-γ ELISpot assay and irrespective of the stimulating antigens, the areas under the curve all resembled a random predictor. In **chapter 2**, it was also shown that more than half of the treated LNB patients reported non-specific symptoms, and these symptoms could not be linked to IFN-γ ELISpot reactivity. IFN-γ ELISpot reactivity among treated healthy individuals could also not be linked to symptoms, as healthy individuals were only included when they did not report any complaints. Similarly, no association was found between the presence of symptoms and IFN-γ ELISpot reactivity as determined by the commercial LymeSpot assay in **chapter 3**. Therefore, it was concluded that the IFN-γ ELISpot reactivity among both treated groups is most likely explained by a previous, yet cleared, LB, and that the IFN-γ ELISpot assay seems to differentiate between *Borrelia*-naïve and *Borrelia*-infected individuals. The findings in **chapters 2** and **3** are

in line with those found using the IFN-γ ELISpot assay for TB as this assay cannot discriminate between active and latent TB [75].

FACTORS INFLUENCING THE LIMITED REACTIVITY OF THE IFN-γ ELISPOT ASSAY AMONG ACTIVE LNB PATIENTS

Overall, the IFN-γ ELISpot assay suffered from a low or even absent IFN-γ ELISpot reactivity among active LNB patients. Several factors may influence the limited reactivity of the IFN-γ ELISpot assay among patients with active LNB, such as: (i) the type and number of antigen(s) or strain(s) and/or the antigen concentration used to stimulate the PBMCs, (ii) the inflammatory marker that is being measured after stimulation of the PBMCs, or (iii) the patient sample (whole blood versus CSF) used to isolate the PBMCs from. All these factors will be discussed below.

CHOOSING THE ANTIGEN(S)

The first factor that may explain the limited reactivity of the IFN-γ ELISpot assay is the antigen used to stimulate the PBMCs. Both the type and number of antigen(s) and/or strain(s) as well as the antigen concentration used to stimulate the PBMCs can influence the IFN-γ ELISpot reactivity. A low or absent IFN-γ ELISpot reactivity can be found if the antigens used to stimulate the PBMCs do not match the antigens expressed by the strain causing disease. When a whole-cell lysate is used, the composition of the expressed antigens of a cultured strain can differ from the antigens expressed in humans during active disease, as some antigens are only expressed in vivo or are lost during multiple culture passages [83, 84]. Consequently, a discrepancy can occur between the expressed antigens of a (cultured) whole-cell lysate that is used in the IFN-γ ELISpot assay and the antigens expressed during active disease and against which the T-cell immune response is directed. Also, different *Borrelia* species can cause LNB, and these can also differ in the antigens that are expressed [25].

One of the antigens used in the IFN-γ ELISpot assays under investigation in this thesis is a whole-cell lysate of *Borrelia burgdorferi* s.s. strain B31 (hereafter: *B. burgdorferi* B31). This strain is isolated from an *Ixodes dammini* (now: *Ixodes scapularis*) tick in North America [85]. In Europe, as well as in the Netherlands, LNB is mainly caused by *Borrelia garinii* and *Borrelia bavariensis* (previously known as *B. garinii* OspA type 4 [86]), and less frequently by *Borrelia afzelii* and *Borrelia burgdorferi* s.s. [85, 87-91]. Therefore, it could be hypothesized that the strain used in the IFN-γ ELISpot assay is not capable of inducing a T-cell response when the patient is infected with another *B. burgdorferi* s.l. strain. This hypothesis might be endorsed by two studies investigating the QuantiFERON ELISA, an IFN-γ assay that also uses *B. burgdorferi* B31. The first study, conducted in North America, showed a sensitivity of approximately 70% [92] for this assay among EM patients, and this sensitivity was much higher than the sensitivity found among LB patients in the second study, which was performed in the Netherlands (i.e., the sensitivity did not exceed 10%) [93].

Speaking against this hypothesis are the results of a pilot study performed in our laboratory, of which the results have not been published. In this pilot study, whole-cell lysates of *B. garinii, B. afzelii,* and *B. burgdorferi* B31 were evaluated using the in-house *Borrelia* ELISpot assay. In total, 49 cases (one active LNB patient, 15 treated LNB patients, eight healthy individuals treated for an early (mostly cutaneous) manifestation of LB in the past and 25 untreated healthy individuals) were included. For most of the cases who showed increased IFN-γ ELISpot reactivity, the results using whole-cell lysates of *B. garinii* and *B. afzelii* were inferior compared to that of a whole-cell lysate of *B. burgdorferi* B31. Nordberg et al. [94] also evaluated the use of Osps derived from a *B. garinii* strain in an IFN-γ ELISpot assay using CSF instead of blood, and found a sensitivity of 36% and a specificity of 82%. They concluded that the IFN-γ ELISpot on CSF is not useful for LNB diagnostics. Widhe et al. [95] also evaluated the IFN-γ ELISpot assay using different (overlapping)

peptides of *B. garinii* OspA on CSF and blood and showed that CSF, which was superior to blood, had moderate sensitivities (range: 38% to 50%). Forsberg et al. [96] evaluated the IFN-γ ELISpot assay using different fractions of *B. afzelli* on CSF and blood. They showed that CSF was superior to blood, that the Osp fraction (OspA and OspB) was superior compared to the flagellar fraction, and that a more heterogeneous sonicated fraction was reactive among both LNB patients and controls. The choice of antigen, thus, appears to be important. This is in line with the results found in this thesis, as the Osp-mix, consisting of a pool of 9-mer to 11-mer peptides of Osp-A (*B. burgdorferi*, *B. afzelii* and *B. garinii*), native Osp-C (*B. afzelii*) and recombinant p18, was inferior to *B. burgdorferi* B31 (**chapters 2** and **3**). Despite the aforementioned difficulties, it is expected that a whole-cell lysate of *B. burgdorferi* B31 will induce a T-cell response in LNB patients, even if these patients are infected with a different *B. burgdorferi* s.l. strain and/or different antigens are expressed in vitro versus in vivo. A measurable T-cell response is expected given the high degree of similarity between the various *Borrelia* genospecies that can cause LNB, and given the number of different antigens that can elicit an immune response. The antigens expressed by the *B. burgdorferi* B31 that was used will, therefore, most likely be cross-reactive with those expressed by the infecting strain.

As mentioned previously, the use of a whole-cell lysate of *B. burgdorferi* B31 in the IFN-γ ELISpot assay is superior to the use of an Osp-mix (**chapters 2** and **3**). This may be caused by a mismatch of the antigens present in the Osp-mix with those expressed by the infecting strain. It may also be caused by the (limited) number of antigens present in the Osp-mix, and/or the antigen concentration in this mix, which may be too low. The difference in IFN-γ ELISpot reactivity using the Osp-mix (lower) or *B. burgdorferi* B31 (higher) may, thus, reflect a positive correlation between the number of antigens used to stimulate the PBMCs and the level of IFN-γ ELISpot reactivity, which is in line with other reports [97, 98]. However, if the concentration of the antigens in the Osp-mix is too low, these antigens may fail to elicit an immune response, a phenomenon that is also seen in antibody assays. In another pilot study conducted in our laboratory, culture supernatants of the in-house *Borrelia* ELISpot assay, which had been stored at -80°C after the overnight stimulation of PBMCs with *B. burgdorferi* B31 and/or the Osp-mix, were investigated for the presence of other inflammatory markers that may have been produced. Indeed, other inflammatory markers were elevated among patients with LNB. The level of these markers, however, were much lower in the Osp-mix-stimulated wells than in the *B. burgdorferi* B31-stimulated wells. This suggest that the concentration of antigens in the Osp-mix indeed is too low and should be increased. This hypothesis is supported by a study from Kaiser et al. [99], who showed that increasing the concentration of antigens in an antibody assay that is based on a limited number of antigens indeed was effective and increased the sensitivity of the assay. One could also increase the number of different recombinant antigens that is currently present in the Osp-mix in order to increase the IFN-γ ELISpot reactivity, which has also been effective in antibody assays [100, 101]. Several recombinant antigens to consider, as they have proven beneficial in the serodiagnosis of LNB, are decorin binding protein (Dbp)A and VlsE, which are only expressed in vivo, and BBK32 [100-103]. Especially VlsE would be interesting, as this antigen is the most sensitive antigen for the detection of IgG [101] and warrants further investigation.

What is also important to consider is the ability of the *Borrelia* bacterium to change its surface antigens, or its ability to escape or suppress the immune system, as this may also explain the absence of IFN-γ ELISpot reactivity among active LNB patients [104-106]. However, as mentioned above, the number of antigens that can elicit an immune response using a whole-cell lysate is numerous. Therefore, this hypothesis does not seem likely, also since the sensitivity of detecting *Borrelia*-specific antibodies in serum of patients with active LNB using a whole-cell lysate is very high as was shown in **chapter 7**. In this chapter, all active LNB patients (all of them were also part of the study population used in **chapter 2**)

had *Borrelia*-specific antibodies in serum for which a screening test based on a whole-cell lysate (with recombinant VlsE added to the IgG ELISA) was used and confirmation was done using an immunoblot based on multiple recombinant antigens.

CHOOSING THE MARKER(S)

A second factor that may explain the limited reactivity of the IFN-γ ELISpot assay is the possibility that IFN-γ may not be the best marker to measure the activity of the cellular immune response upon infection with *B. burgdorferi* s.l.. It would be worth investigating if other makers are more suitable. Preliminary results from our previously mentioned pilot study on culture supernatants of the in-house *Borrelia* ELISpot assay, which were investigated for the presence of other inflammatory markers, seem promising. Some of the investigated markers, including the B-cell chemokine (C-X-C motif) ligand 13 (CXCL13), differed between active LNB patients and controls and warrants further investigation. In the literature, the potential role for LNB diagnostics of other cytokines and/or chemokines in blood [107, 108], CSF [10, 106, 109-111] or both [112] has been shown. During the course of our studies, the manufacturer of the commercial LymeSpot assay has adapted their assay by adding the detection of interleukin (IL)-2, which has shown promising results in TB diagnostics [113-115]. However, at the time of writing, no data are available yet with regard to the diagnostic performance of this modified LymeSpot assay.

CHOOSING THE PATIENT SAMPLE

A third factor that may explain the limited reactivity of the IFN-γ ELISpot assay is the patient sample that is chosen to isolate the PBMCs from. The immune response against *B. burgdorferi* s.l. among LNB patients is suggested to be compartmentalized within the central nervous system (CNS) [95, 116, 117]. This is in line with several reports that suggest that *B. garinii* disseminates into the CSF via migration along the peripheral nerves [105, 118]. This so-called compartmentalization within the CNS might explain the much lower IFN-γ ELISpot reactivity in blood among LNB patients than the IFN-γ ELISpot reactivity in blood among patients with TB, cytomegalovirus, or Q-fever using an IFN-γ ELISpot assay for these respective diseases [119-122]. Indeed, several reports showed that more IFN-γ ELISpot reactivity was seen in the CSF than in the blood of LNB patients [95, 96], as was mentioned previously. Yet, at the start of our research project in 2011, a pilot study was performed using the in-house *Borrelia* ELISpot assay on CSF of LNB patients, which was unsuccessful as the CSF-leucocyte count was often too low to be used in the IFN-γ ELISpot assay (i.e., <100,000 PBMCs), even in active LNB patients. This precluded the use of CSF with this assay as no, or limited, IFN-γ ELISpot reactivity was seen, even in proven cases. After several try outs, it was decided that the use of CSF would not work, and the focus was directed on performing the in-house *Borrelia* ELISpot assay on blood instead of putting more effort in miniaturizing the IFN-γ ELISpot assay for application with CSF-leucocyte counts that are too low to be used in this assay. As the majority of the LNB patients in this thesis developed *Borrelia*-specific antibodies in blood, we do not believe that the immune response against *Borrelia* is solely located in the CNS. This hypothesis is further supported by the results of our pilot study on culture supernatants of the in-house *Borrelia* ELISpot assay mentioned previously. Furthermore, the potential role for LNB diagnostics of other cytokines and/or chemokines in blood has also been reported in literature [107, 108, 112].

FACTORS INFLUENCING THE REACTIVITY OF THE IFN-γ ELISPOT ASSAY AMONG CONTROLS

Besides the limited reactivity of the IFN-γ ELISpot assay among active LNB patients, increased IFN-γ ELISpot reactivity was seen among two of the three control groups in **chapters 2** and **3**. In both chapters, the IFN-γ ELISpot reactivity was more pronounced among treated LNB patients and treated healthy individuals than among untreated healthy individuals. The B-cell response, however, was comparable between the three control groups (i.e., the presence of *Borrelia*-

specific serum antibodies in these groups ranged from 12% to 18% [**chapter 2**], and from 8% to 40% [**chapter 3**]). Indeed, IFN-γ ELISpot reactivity in each of the three control groups could not be linked to seropositivity. Several arguments could underlie these findings.

Firstly, IFN-γ ELISpot reactivity in the absence of *Borrelia*-specific antibodies among controls can suggest an active, asymptomatic infection. Greissl et al. [123] showed that the cellular immune response preceded the antibody response in more than one-third of patients (*n* = 211) with early LB. These results are in line with a report from Dattwyler et al. [29], who also found a strong proliferative T-cell response against *B. burgdorferi* s.l. in the absence of *Borrelia*-specific serum antibodies among 17 Lyme patients. Thus, IFN-γ ELISpot reactivity in the absence of *Borrelia*-specific antibodies among treated LNB patients, treated healthy individuals and untreated healthy individuals in our study may reflect an active, asymptomatic infection. Perhaps this is most likely among (un)treated healthy individuals, as they were invited to participate if they engaged in recreational activities in high-risk areas for tick bites. For some of the (un)treated healthy individuals in **chapter 2**, a second blood sample had been drawn at a later time point (data not shown). In total, 17 (un)treated healthy individuals who were seronegative at inclusion in the study, were also seronegative using the follow-up sample (median number of days between both samples was 700 (range 113-741 days) (data not shown in **chapter 2**). Interestingly, for most of them, the IFN-γ ELISpot reactivity seen at inclusion in the study, had considerably decreased at this later time point (median *B. burgdorferi* B31-specific SFC-count: 13.6 [range: 1.0 to 97], and 4.3 [range: 0.0 to 53], respectively) (data not shown in **chapter 2**), and warrants further investigation.

Secondly, IFN-γ ELISpot reactivity in the absence of *Borrelia*-specific antibodies among controls can also be explained by cross-reactivity. The *B. burgdorferi* B31 whole-cell lysate that was used in both IFN-γ ELISpot assays evaluated in this thesis contains epitopes that are shared by other related spirochaetal micro-organisms. For serology, false-positive test results have been found in patients infected with treponemes [124-128], leptospires [128], relapsing fever *Borrelia* [128-130], or *Helicobacter pylori* [126]. Limited data are available with regard to false-positive test results using IFN-γ ELISpot assays [131]. In **chapter 2**, we discussed that one of four patients with potential cross-reacting diseases (either leptospirosis (n=2) or neurosyphilis (n=2)) had increased IFN-γ ELISpot reactivity and this patient was diagnosed with active leptospirosis. The T-cell response among controls may, therefore, also be explained by cross-reactivity.

Thirdly, IFN-γ ELISpot reactivity in the absence of *Borrelia*-specific antibodies may imply that the *Borrelia*-specific B-cell response wanes faster than the *Borrelia*-specific T-cell response. Most (30/36; 83.3%) of the treated LNB patients in **chapter 2**, for instance, all of whom were seropositive when diagnosed with LNB in the past, had seroreverted at the time of inclusion in the study. Similar results were found by Greissl et al. [123], who reported a faster decline of the antibody response than that of the cellular immune response using T-cell receptor sequencing on blood from patients treated for early Lyme disease.

THE IFN-γ ELISPOT ASSAY MIGHT HAVE A POTENTIAL ROLE FOR DIAGNOSING POSSIBLE LNB PATIENTS

Even though the IFN-γ ELISpot assay does not seem useful for LNB diagnostics, it might still be of added value among possible LNB patients. In **chapter 2**, one quarter (8/33) of the active LNB patients were classified as possible LNB patient based on the presence of pleocytosis and the absence of intrathecally produced *Borrelia*-specific antibodies [6]. Half of them (*n* = 4) showed a marked IFN-γ ELISpot reactivity (i.e., the *B. burgdorferi* B31-specific SFC-counts ranged between 13 and 34, with a median SFC-count of 6.0 [interquartile range: 0.5 to 14]) (data not shown in **chapter 2**). For these four cases, the cellular immune response might have preceded the humoral

response, although this could not be confirmed, as a second LP was never done. It is, however, supported by a negative correlation between the level of IFN-γ ELISpot reactivity and the level of intrathecally produced *Borrelia*-specific IgM among active LNB patients with a positive *Borrelia*-specific IgM AI result.

As was mentioned earlier, it has also been postulated that possible LNB patients with pleocytosis represent early LNB cases and that possible LNB patients with intrathecal *Borrelia*-specific antibody synthesis most likely have had a previous LNB or another disease [42, 50]. The four possible LNB patients with pleocytosis and a marked IFN-γ ELISpot reactivity, therefore, most likely had early LNB. For two of them, the CSF-CXCL13 levels had also been determined as they were also part of the study population described in **chapter 4**. One of them had a positive CSF-CXCL13 result in both CXCL13 assays; the other patient had elevated CSF-CXCL13 levels in both assays; however, these levels were below the assay-specific cutoff value. This patient was the one who had paresis of the VIIth nerve, reported a tick bite 3 weeks earlier, had symptoms for 5 days, and responded well to antibiotic treatment for LNB. Therefore, it can be assumed that the CSF-CXCL13 concentration for this patient was building up at the moment of the LP. Based on the IFN-γ ELISpot reactivity among some of the possible LNB patients with pleocytosis in the absence of detectable levels of intrathecally produced *Borrelia*-specific antibodies, the IFN-γ ELISpot could be of added value in diagnosing active LNB, although the IFN-γ ELISpot reactivity should be interpreted with care as reactivity was also seen among controls (**chapters 2** and **3**) [94, 132, 133].

CORRELATION OF THE ABSENCE OF A TICK BITE AND MALE GENDER WITH LNB

In **chapter 2**, it was shown that patients who did not report a tick bite were 2.9 times more likely to have LNB, which was significant ($P = 0.029$), and males were 2.1 times more likely to have LNB, which was not significant ($P = 0.106$). As expected, considering the results of the ROC analysis, IFN-γ ELISpot reactivity did not contribute at all. The overrepresentation of the male gender amongst LNB patients is in line with various published reports showing a male predominance ranging from 56% to 65% [134-139]. The increased odds of having LNB in the absence of a tick bite also seems reasonable, as not noticing a tick bite may increase the chances of developing disseminated LB. Also, it is expected that individuals who do notice a tick bite are more keen on developing LB symptoms. Consequently, these individuals will more likely seek medical advice if they do develop such symptoms. They are, therefore, less likely to develop disseminated LB. In general, the report of a recent tick bite often contributes to the diagnosis of LB [140]. However, the absence of a tick bite clearly does not rule out LB, especially in endemic regions or when displaying risk behavior, and should be taken into consideration, especially among cases with presumed symptoms of disseminated LB. This observation stresses the importance of carefully inspecting the body for ticks after outdoor activities.

FUTURE PERSPECTIVES

Much of the research conducted in this thesis was possible due to setting up the T-cell response in Lyme (TRIL)-study in 2011. This study has been set up in order to investigate the T-cell response to *Borrelia*-specific antigens using the IFN-γ ELISpot assay among patients with LB; however, other parts of the immune system that involve the T- and B-cell response are also investigated. In addition, all participants are asked to fill in two questionnaires: (i) a Lyme-specific questionnaire, including questions regarding tick bites, presence of EM, antibiotic treatment for LB and (self-reported) complaints at the moment of inclusion and during possible earlier episodes of LB, and (ii) the 36-item Short-Form Health Survey (RAND-36) covering the physical and mental health by investigating eight health domains [141]. All active LB patients are currently followed for up to 48 months after first inclusion in the TRIL-study at the moment of active disease. **Chapters 2** and **3**,

in which two ELISpot assays were evaluated for use in LNB diagnostics, are the first published articles using data obtained within the framework of this study.

At the start of the TRIL-study, our focus was on the inclusion of active LNB patients, as active LNB served as a proxy for active disease. As it is difficult to extrapolate the results found among active LNB patients to other Lyme manifestations, active Lyme arthritis patients have been asked to participate in the TRIL-study since 2015, and patients with acrodermatitis chronica atrophicans since 2020. A major component of the TRIL-study is that patients are followed in time for 4 years to gather data on the immune response and perceived health status. Using these data, we hope to gain more knowledge about the T- and B-cell response among LB cases at the moment of active disease as well as over time. Furthermore, we hope to gain insight into the circumstances that lead to the persistence of symptoms among a subset of patients treated for active LB by relating reported symptoms with the T- and B-cell immunity as well as with host-genetic factors.

At present, the IFN-γ ELISpot assay cannot be used in routine clinical practice for LNB diagnostics. More research is necessary and the results of the VICTORY study initiated with funding from the Government by the instigated Lyme expertise center [142], in which a large cohort of patients with a variety of Lyme manifestations are tested using different cellular assays including two IFN-γ assays, are to be expected soon. The results of our studies described in **chapters 2** and **3** suggest that IFN-γ may not be the best marker to diagnose active LNB. It would be interesting to investigate whether other cytokines and/or chemokines could improve the performance of the IFN-γ ELISpot assay, especially since the clinical symptoms of LNB for a large part seem to be attributable to the effects of the hosts' innate and adaptive immune response [143]. For TB, IFN-γ release assays cannot differentiate between active and latent TB [144, 145], but when other cytokines are included, such as tumor necrosis factor (TNF)-α, a difference between active and latent TB was seen [146]. A recent publication also showed that both of these disease stages can be characterized by a certain immunological signature consisting of various cytokines, chemokines and enzymes [147]. Just recently, Ogrinc et al. [55] showed that LNB patients with meningoradiculoneuritis and LNB patients with peripheral facial palsy varied in innate and adaptive immune response. Based on the results of our pilot study on the culture supernatants of the in-house *Borrelia* ELISpot assay, and supported by several reports in the literature [10, 107-112, 148], other cytokines and/or chemokines might be more suitable for LNB diagnostics, and an expansion of our pilot study is already planned for the near future. Furthermore, it will be interesting to characterize the *B. burgdorferi* s.l. strain causing LNB in individual cases as different *B. burgdorferi* s.s. OspC types are associated with more severe inflammation and disease in case of Lyme arthritis [48]. Likewise, it is interesting to investigate host genetic factors as certain polymorphisms in human genes have also been associated with excessive immune responses and more symptomatic disease [49, 55]. Finally, metabolomics might be a useful tool to differentiate patients that recover following antibiotic treatment from those that experience longer lasting symptoms [149].

Furthermore, a large prospective, multicenter study should be conducted to evaluate the diagnostic algorithm found in **chapter 6**, especially since the number of LNB patients included in this study was limited due to the cross-sectional study design that was chosen. Such a multicenter study should aim at including more LNB patients in a joint effort to provide a robust diagnostic tool, with high sensitivity and specificity, that should - ideally - be universally applicable. The main focus for this multicenter study should be on including early LNB patients, as these patients are more difficult to diagnose with current available tests [3, 22], as was also shown in this thesis. The inclusion of early LNB patients may be challenging as the immune response of these patients is building up, and is often too low to be measurable. Expansion of the diagnostic algorithm with other (new) markers, which may be detected in the culture supernatants of the in-house *Borrelia* ELISpot assay, should also be investigated.

The sole use of the direct detection of (non-specific) inflammatory markers for LB diagnostics may not be sufficient, and *Borrelia*-specific tests should always be part of the diagnostic work-up. Ideally, tests should be easy to perform and have high sensitivity and specificity. T-cell receptor sequencing has shown promising results for use in diagnostics of various other diseases [150-152] and is worth investigating in future studies as well. The potential role of CSF-CXCL13 has already been shown, but more research is warranted to decipher the underlying causes of different cutoff values and on investigating the possibilities of defining an international reference standard and/or a setting-specific cutoff value that - preferably - is independent of the assay used.

CONCLUSION

To return to the aims of this thesis and the challenges medical specialists have to face surrounding LNB diagnostics, we believe that the results in this thesis are promising, and that the construction of a diagnostic algorithm that includes different aspects of the immune system (innate and adaptive, B- and T-cell response) will help to solve some of these challenges and more clearly discriminates active disease from a past infection.

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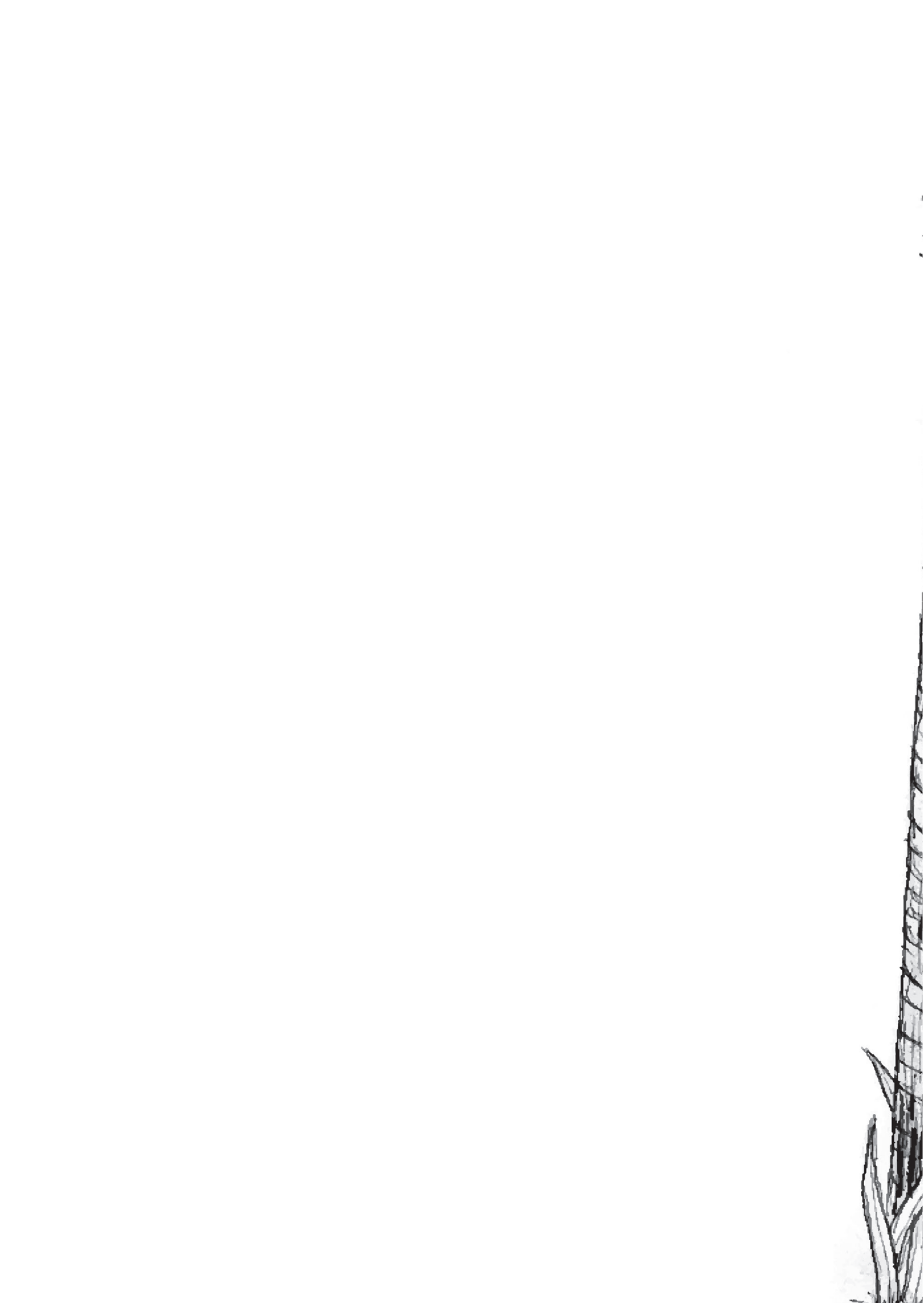

9 SUMMARY

Lyme disease, also known as Lyme borreliosis (LB), is caused by spiral-shaped bacteria, so-called spirochetes, which are part of the *Borrelia burgdorferi* sensu lato (s.l.) complex group [1]. The three most prevalent *B. burgdorferi* s.l. species in Europe are: *Borrelia afzelii*, *Borrelia garinii* and *Borrelia burgdorferi* sensu stricto (s.s.), while in North America the predominant species is *B. burgdorferi* s.s. [2]. *B. burgdorferi* s.l. is transmitted by a bite from an infected *Ixodes*-tick, which is mainly found in temperate regions in the Northern Hemisphere [3]. The most prevalent manifestation of LB in the early phase of the infection is characterized by a red, migrating skin lesion, also known as erythema migrans (EM) [2]. If left untreated, then *B. burgdorferi* s.l. can disseminate through the body and infect other body parts such as the joints (Lyme arthritis), the nervous system (Lyme neuroborreliosis (LNB)), the heart (Lyme carditis) and/or other parts of the skin (acrodermatitis chronica atrophicans) [2].

The diagnosis of LB is mainly based on the presence of clinical symptoms. The classical EM lesion is sufficient for a clinical diagnosis [4, 5]. However, in case of unclear skin lesions and/or other Lyme manifestations, laboratory tests are needed to support and confirm the clinical diagnosis [4, 5]. These laboratory tests are based on the direct or indirect detection of *B. burgdorferi* s.l. [6]. Direct detection methods include microscopy, culture, or PCR; however, since the bacterium is usually present in low numbers, direct detection methods are hampered by a low sensitivity [6]. Except for PCR on skin biopsy samples (e.g., in case of symptoms consistent with EM or acrodermatitis chronica atrophicans) or synovial fluid (e.g., in case of symptoms consistent with Lyme arthritis) [6]. Indirect detection methods are based on the host's immune response against *B. burgdorferi* s.l., most importantly the detection of *Borrelia*-specific antibodies [6].

The diagnosis of active LB can be challenging as symptoms are often difficult to interpret and/or not specific for LB [4]. The interpretation of laboratory tests can also be challenging: a negative antibody test result is no proof of the absence of active LB, and a positive antibody test result is no proof of active LB [7]. Because of these diagnostic challenges, the research in this thesis focuses on whether current the diagnostics for LB can be improved, and whether an active infection can be distinguished from a past infection. To answer these questions, current and alternative diagnostic tests and/or algorithms are evaluated, including well-known diagnostic tests based on the humoral immune response (i.e., the detection of *Borrelia*-specific antibodies) and alternative diagnostic tests based on the cellular immune response (i.e., the detection of certain signaling molecules produced by so-called T cells). For this research, a study population has been established consisting of well-defined patients and controls.

As clear criteria are defined for the diagnosis of patients with LNB, we mainly focused on these patients. Patients have LNB if they fulfil at least two of the three following criteria defined by the European Federation of Neurological Societies (EFNS) [8]:

1. Presence of neurological symptoms suggestive of LNB without another obvious reason
2. Elevated cerebrospinal fluid (CSF) cell count (≥5 leukocytes/µl CSF [i.e., pleocytosis])
3. Intrathecal synthesis of *Borrelia*-specific antibodies

If a patient fulfils all criteria, then the patient is classified as definite LNB; if a patient fulfils two of the three criteria (including the presence of neurological symptoms suggestive of LNB without another obvious reason), then the patient is classified as possible LNB.

The detection of intrathecally produced *Borrelia*-specific antibodies is, thus, important in LNB diagnostics. Many commercial antibody tests are available; however, the performance characteristics of these tests are often difficult to interpret due to variability in the study designs, heterogeneity of the study populations under investigation, and poor reporting of study characteristics [9]. Ideally, evaluation of tests is done using a prospective, cross-sectional study

design in a setting where the test will be used in clinical practice. However, case-control study designs are used more often, because these are easier to perform, despite that such a study design has a risk of introducing bias [9]. This bias often occurs because the control group does not represent the same clinical background as the patient group. Consequently, the obtained results are not representing reality [9].

The most important finding of the research conducted in this thesis are the promising results described in **chapter 6**, as these will aid clinicians in diagnosing LNB. In this chapter, seven antibody tests for LNB diagnostics were evaluated. Therefore, a cross-sectional study design was used in which all consecutive patients of the neurology department, from whom a CSF-serum sample pair was sent to the microbiology department of the Diakonessenhuis Hospital during a certain timeframe, were retrospectively included. For each antibody test, a multiparameter analysis was conducted to investigate whether the diagnostic performance of the antibody test could be further improved by including additional parameters. These additional parameters included various routine CSF parameters (i.e., pleocytosis, total protein, blood-CSF barrier functionality and intrathecal total-antibody synthesis), a *Borrelia*-specific (i.e., *Borrelia* species PCR) and a non-specific CSF parameter (i.e., B-cell chemokine (C-X-C motif) ligand 13 [CXCL13]), and a *Borrelia*-specific serum parameter (i.e., *Borrelia*-specific serum antibodies). The results of these multiparameter analyses show that for most of the antibody assays, the use of additional parameters for the diagnosis of LNB results in higher sensitivities (range: 94.1% to 100%) and slightly lower specificities (range: 92.8% to 96.4%) than the sensitivities and specificities of the individual antibody tests (range: 47.1% to 100 % and 95.7 % to 100%, respectively). The most important parameters that contribute to improved LNB diagnostics are intrathecally produced *Borrelia*-specific antibodies, blood-CSF barrier functionality, intrathecal total-antibody synthesis, pleocytosis, CSF-CXCL13, and *Borrelia*-specific serum antibodies. Even though other studies have also shown the additional value of these parameters in LNB diagnostics [8, 10-12], our study is the first that shows the relative importance of these parameters. Furthermore, our study shows which parameters contribute the most and, consequently, are suitable to be added to a diagnostic algorithm for LNB diagnostics. Our study also shows that measurement of both the humoral and the cellular immune response against an infection with *B. burgdorferi* s.l. contribute to the diagnosis of LNB, and that individual parameters can either be (false) negative or (false) positive, and still be of added value in the broader context. It is our opinion that these results merit a multi-center validation. Therefore, we propose an (inter)national prospective study to investigate the potential use of a standardized diagnostic algorithm for LNB based on multiparameter analysis.

Overall, the results of the multiparameter analyses in **chapter 6** show that, of the included parameters, the detection of intrathecally produced *Borrelia*-specific antibodies contribute the most in LNB diagnostics. For the detection of pathogen-specific antibodies an enzyme-linked immunosorbent assay (ELISA) is often used [4]. ELISAs, however, can suffer from a so-called 'edge effect' [13]. An edge effect is found when the measurements of a single antibody concentration in wells located at the edges of an ELISA plate are higher (or lower) than the measurements in wells located in the center of an ELISA plate. If an ELISA plate suffers from an edge effect, then this may have consequences for the patient samples tested in this plate. In **chapter 5**, it is shown that the commercial Enzygnost Lyme link VlsE/IgG ELISA suffers from an edge effect. It is also shown that the impact of the edge effect on LNB diagnostics using this ELISA could be reduced by some minor adaptations to the standard protocol of the manufacturer. This adapted protocol was subsequently used to test 149 CSF-serum sample pairs which were part of the slightly larger sample set used in **chapter 6** (and **4**). By simulation it was shown that if the standard protocol of the manufacturer would have been used, then the edge effect for this study population in a 'worst-case' scenario could have resulted in 15 (10.1%) false-positive and two (1.3%) false-negative *Borrelia*-specific IgG antibody index results. The observed edge effect can, thus, lead

to inaccurate LNB diagnostics. The results in **chapter 5** underline the importance of a thorough validation of ELISAs before use in routine diagnostics as is required, for instance, by the ISO 15189 accreditation [14].

The diagnosis of most of the other Lyme manifestations is supported by the detection of *Borrelia*-specific antibodies in blood, for which a two-tier test strategy is recommended [5, 15]. This two-tier test strategy aims to improve the diagnostic performance of laboratory tests by combining a highly sensitive first test (i.e., a screening test) with a highly specific second test (i.e., a confirmation test) to confirm equivocal and positive test results obtained in the first test [7, 16, 17]. The screening test often comprises an ELISA, and the confirmation test is based on either a western blot (North America) or an immunoblot (Europe) [7]. Antibody tests can vary in sensitivity, as is shown in **chapter 6**, which can result from a mismatch between the antigens applied in the assay and those expressed by the *Borrelia* bacterium during an active infection. This discrepancy can be caused by the intra- and interspecies heterogeneity of *B. burgdorferi* s.l [11, 18-22] and/or antigenic variation used by the infecting strain during the course of the disease [23]. The sensitivity of an antibody test can also be influenced by early antibiotic treatment, as this can abrogate the immune response [24-28]. This abrogation seems to be antibody-specific, as antibodies against the C6-peptide wane faster than those against a whole-cell lysate of *B. burgdorferi* s.l. [29, 30] or protein (p)39 [29].

In **chapter 7**, two screening tests (i.e., the C6 ELISA and the Serion ELISA) and two two-tier test strategies (confirmation of equivocal and positive C6 ELISA and Serion ELISA test results using the *recom*Line immunoblot) for the detection of *Borrelia*-specific serum antibodies were evaluated. The research in this chapter shows that antibiotic treatment of an infection with *B. burgdorferi* s.l is highly associated with discordant screening test (ELISA) results and two-tier test strategy (ELISA + immunoblot) results (odds ratio [OR]: 10.52; $P < 0.001$ and OR: 9.98; $P = 0.014$, respectively). This suggests that antibiotic treatment influences the pace at which the different *Borrelia*-specific antibodies wane, as antibodies against the C6 peptide appear to wane faster than those against whole-cell lysates of *B. burgdorferi* s.l., which confirm previous findings [29, 30]. Most of the discordant test results in our study were explained by the presence of IgM against outer surface protein (Osp)C, and for some test results also against p41 flagellin, as was also shown in earlier studies [31, 32]. These results underline the challenges with regard to the interpretation of antibody tests which should be taken into account when such tests are used for the diagnosis of LB.

In the early phase of an infection, antibody levels are still too low to be detected as the immune response has to be build up. Consequently, there is a need for markers of infection with high sensitivity and specificity, especially in the first few weeks after infection. In the last two decades, various studies have been published that have shown the added value of elevated levels of CXCL13 in the CSF of patients with early LNB [33, 34]. The detection of CXCL13 in the CSF is relatively easy in contrast to the often complex calculations needed to proof the intrathecal synthesis of *Borrelia*-specific antibodies. In **chapter 4**, two commercial CXCL13 tests (the Quantikine CXCL13 ELISA and the *recom*Bead CXCL13 test) on CSF for LNB diagnostics were evaluated. Using the same study population as the one in **chapter 6** (and almost the same study population as the one in **chapter 5**), it is shown that measuring the CXCL13 level in CSF is of added value for the diagnosis of patients with active LNB. It was also shown that CXCL13 can be detected in the CSF prior to intrathecally produced *Borrelia*-specific antibodies, and that measuring CXCL13 in the CSF is especially useful in the diagnosis of early LNB. The cutoff values for both tests, however, differ and this might be caused by differences in methodology [35]. Also in the literature, different cutoffs are reported for the same tests [35-41]. Therefore, more research is needed to elucidate the reasons behind these differences and to

investigate whether an international reference standard for CXCL13 in the CSF can be established.

The production of CXCL13 in the CSF is part of the cellular immune response. The cellular immune response against *B. burgdorferi* s.l. is characterized by a strong T helper (Th)1 response in which T cells will produce Th1-cytokines, among which interferon-gamma (IFN-γ). In the past years, other tests that focus on the cellular immune response have also become available. One of these tests is the enzyme-linked immuno spot (ELISpot) test in which *Borreli*a-specific IFN-γ producing T cells are detected. In **chapters 2** and **3**, two of such tests for LNB diagnostics were evaluated: an in-house *Borrelia* ELISpot test and the commercial LymeSpot test. Many Lyme patients go to Germany, where commercial IFN-γ ELISpot tests are used, even though the clinical validation of these tests on well-defined patient populations are lacking. Yet, when the IFN-γ ELISpot test result for these patients is positive, often (long-term) antibiotic treatment is given [42]. For the evaluation of the two IFN-γ ELISpot tests in this thesis, a prospective 'case-control' study was used comprising of active LNB patients (i.e., cases), and three control groups comprising treated LNB patients, and healthy individuals with - and without - a history of treated LB (mainly cutaneous). The studies in **chapters 2** and **3** show that both IFN-γ ELISpot tests cannot be used to prove active LNB. The presence of *Borrelia*-specific T-cell reactivity was comparable between active LNB patients, treated LNB patients and treated healthy individuals, and this reactivity was higher than the *Borrelia*-specific T-cell reactivity among untreated healthy individuals. The elevated *Borrelia*-specific T-cell reactivity among both treated groups is most likely explained by a previous, cured LB. The IFN-γ ELISpot test, thus, seems to differentiate between *Borrelia*-naive and *Borrelia*-infected individuals.

CONCLUSION

We believe that the research in this thesis has contributed to the aims of this thesis. The most important contribution of this thesis is the promising result of using multiple parameters for the diagnosis of LNB (**chapter 6**), in which both the humoral and the cellular immune response are important contributors (**chapters 4** and **6**). Combining various parameters into a diagnostic algorithm, in which different aspects of the immune system are covered, provides a concrete and feasible tool to better discriminate between an active infection and a previous infection and will consequently improve LNB diagnostics. We also believe that the construction of a diagnostic algorithm will be of added value for the diagnosis of other Lyme manifestations.

The intra-assay variation of the ELISA investigated in **chapter 5** underlines the need of a thorough validation of diagnostic tests before such tests will be used in routine diagnostics. If all laboratories adhere to this, then it will provide them insight into the (im)possibilities of the test that is used, and this will improve LB diagnostics. In **chapter 7** it is shown that antibiotic treatment influences the humoral immune response, which can explain the differences in test results between different antibody tests. This knowledge will support laboratory specialists as well as medical specialists in the interpretation of antibody tests.

Finally, the thorough evaluation of two IFN-γ ELISpot tests (**chapters 2** and **3**) provides insight into the limitations of the use of these tests for the diagnosis of active LNB and this knowledge is relevant for both medical specialists and patients. Hopefully this can prevent unnecessary antibiotic treatment by incorrect diagnoses, based on IFN-γ ELISpot test results. The results of both studies will also provide a means for medical specialists to talk with patients in the event that these patients have been diagnosed with similar tests elsewhere.

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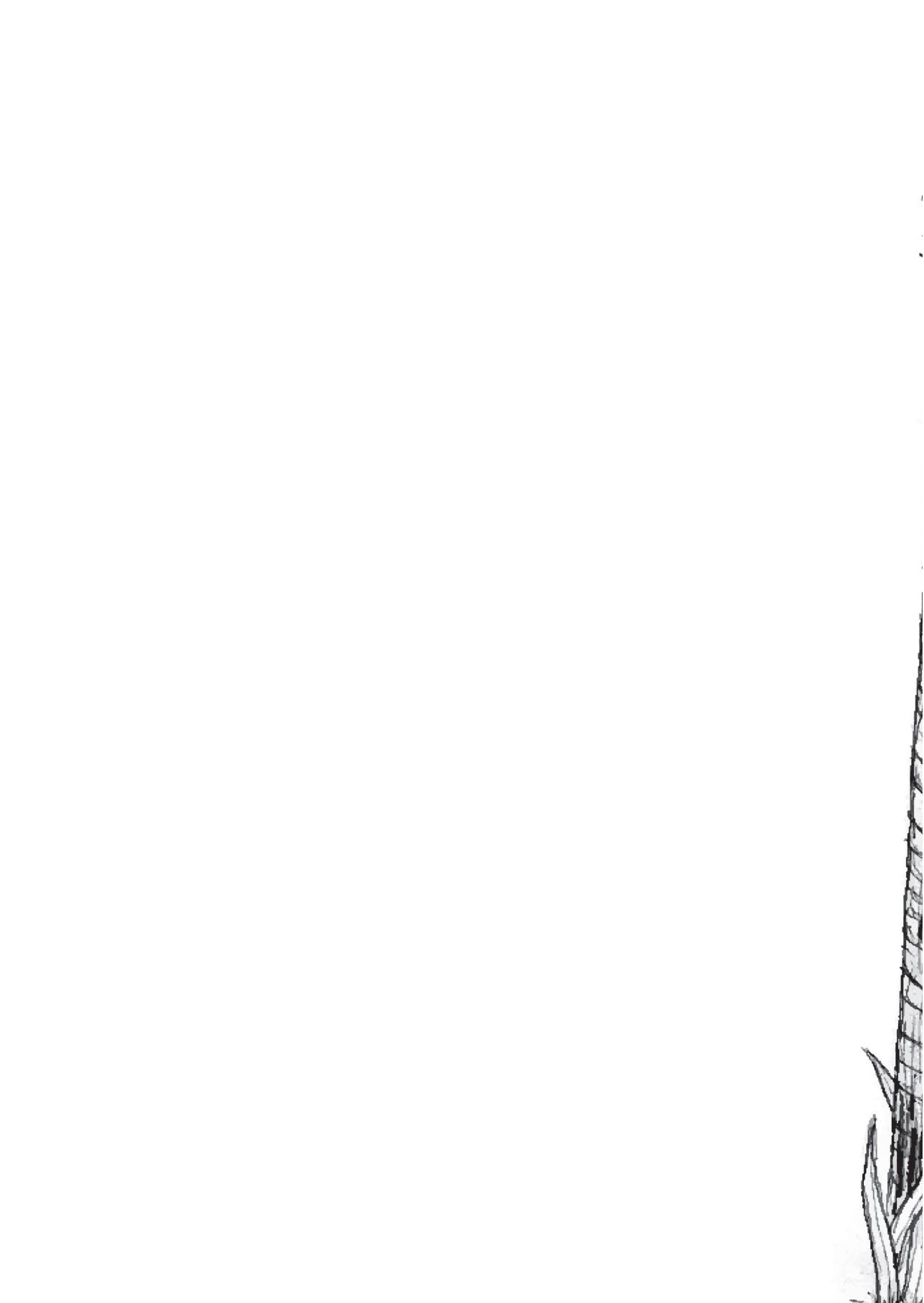

10 NEDERLANDSE SAMENVATTING

De ziekte van Lyme wordt veroorzaakt door spiraalvormige bacteriën, zogenaamde spirocheten, die onderdeel zijn van de *Borrelia burgdorferi* sensu lato (s.l.)-groep [1]. De drie meest voorkomende *Borrelia* species in Europa zijn: *Borrelia afzelii*, *Borrelia garinii* en *Borrelia burgdorferi* sensu stricto (s.s.), terwijl in Noord-Amerika met name *B. burgdorferi* s.s. voorkomt [2]. *B. burgdorferi* s.l. kan op de mens worden overgedragen door de beet van een met *B. burgdorferi* s.l. geïnfecteerde *Ixodes*-teek, welke met name voorkomt in gematigde gebieden op het Noordelijk halfrond [3]. Het meest voorkomende klinische beeld in de vroege fase van de infectie wordt gekarakteriseerd door een rode, zich langzaam uitbreidende huiduitslag, ook wel bekend als erythema migrans (EM) [2]. Indien onbehandeld, kan *B. burgdorferi* s.l. zich door het lichaam verspreiden, met name naar de gewrichten (Lyme artritis), het zenuwstelsel (Lyme neuroborreliose [LNB]), het hart (Lyme carditis) en/of naar andere locaties op de huid (acrodermatitis chronica atrophicans) [2].

De diagnostiek van de ziekte van Lyme richt zich in eerste instantie op de aanwezigheid van klinische symptomen. Het klassieke EM beeld is in principe voldoende om de diagnose te stellen [4, 5]. Is het huidbeeld onduidelijk, of is er sprake van een andere manifestatie van de ziekte van Lyme, dan zijn laboratoriumtesten nodig om de diagnose te kunnen stellen [4, 5]. Deze laboratoriumtesten zijn gebaseerd op het direct of indirect aantonen van *B. burgdorferi* s.l. [6]. Voor het direct aantonen van *B. burgdorferi* s.l. kan gebruik worden gemaakt van microscopie, kweek of PCR, echter, omdat de bacterie vaak in lage aantallen aanwezig is in de mens, is de sensitiviteit van directe detectiemethoden vaak laag [6]. Uitzondering hierop is het gebruik van PCR op huidbiopten (bij symptomen die passen bij EM of acrodermatitis chronica atrophicans) of synoviaal vocht (bij symptomen die passen bij Lyme artritis) [6]. Het indirect aantonen van *B. burgdorferi* s.l. is gebaseerd op de reactie van het immuunsysteem op de bacterie, en dan met name op het aantonen van antistoffen [6].

Het diagnosticeren van actieve Lymeziekte kan uitdagend zijn. Enerzijds zijn de symptomen vaak moeilijk te interpreteren en/of niet Lyme-specifiek [4]. Daarnaast kan de interpretatie van laboratoriumtesten ingewikkeld zijn: een negatieve antistoftest hoeft geen bewijs te zijn voor de afwezigheid van actieve Lymeziekte, en een positieve antistoftest hoeft geen bewijs te zijn voor actieve Lymeziekte [7]. Vanwege deze diagnostische uitdagingen, richt het onderzoek in dit proefschrift zich op de vraag of de huidige diagnostiek van de ziekte van Lyme verbeterd kan worden en of er onderscheidt gemaakt kan worden tussen een actieve infectie en een in het verleden doorgemaakte infectie. Om deze vragen te kunnen beantwoorden zijn huidige en alternatieve diagnostische testen en/of algoritmes geëvalueerd, waaronder veelgebruikte diagnostische testen gebaseerd op de humorale immuunrespons (d.w.z., de detectie van *Borrelia*-specifieke antistoffen) en alternatieve diagnostische testen gebaseerd op de cellulaire immuunrespons (d.w.z., de detectie van bepaalde signaalstoffen geproduceerd door zogenaamde T cellen). Voor dit onderzoek is een studiepopulatie opgezet bestaande uit goed gedefinieerde patiënten en controles.

Omdat er duidelijke criteria bestaan voor het diagnosticeren van patiënten met LNB, hebben we ons met name gericht op deze patiëntengroep. Patiënten hebben LNB als ze voldoen aan ten minste twee van de volgende drie criteria, welke gesteld zijn door de Europese Federatie van Neurologische Verenigingen (European Federation of Neurological Societies [EFNS]) [8]:

1. Aanwezigheid van neurologische symptomen wijzend op LNB zonder een duidelijke andere oorzaak
2. Verhoogde cel aantallen in de liquor (≥5 leukocyten/µl liquor [pleiocytose])
3. Intrathecale synthese van *Borrelia*-specifieke antistoffen

Voldoet een patiënt aan alle criteria, dan krijgt de patiënt de classificatie ‘definitieve LNB’; voldoet een patiënt aan twee van de drie criteria (waaronder in ieder geval de aanwezigheid van

neurologische symptomen wijzend op LNB zonder een duidelijke andere oorzaak), dan krijgt de patiënt de classificatie 'mogelijke LNB'.

De detectie van intrathecaal geproduceerde *Borrelia*-specifieke antistoffen is dus van belang voor de diagnostiek van LNB. Er zijn echter veel verschillende antistoftesten op de markt en de prestatiekarakter-istieken van veel van deze antistoftesten zijn vaak moeilijk te interpreteren vanwege de variabiliteit in studie-opzet, de heterogeniteit van de onderzochte studiepopulaties en de matige rapportage van gebruikte studiekarakteristieken [9]. Idealiter wordt er voor het bepalen van de prestatiekarakteristieken van testen gebruik gemaakt van een prospectieve cross-sectionele studie-opzet in een klinische setting waarin de test uiteindelijk ook gebruikt gaat worden. Desondanks worden er vaker 'case-control' (d.w.z., patiënten versus controles) studies uitgevoerd, omdat deze makkelijker zijn uit te voeren [9]. Echter, dergelijke studies hebben wel een groter risico op gebiaste resultaten. Deze bias wordt verklaard doordat de onderzochte controlegroep vaak niet dezelfde klinische achtergrond heeft als de onderzochte patiëntengroep, waardoor de gevonden resultaten geen goede weergave geven van de werkelijkheid [9].

De belangrijkste bevinding van het onderzoek in dit proefschrift zijn de veelbelovende resultaten beschreven in **hoofdstuk 6**, omdat deze bevindingen artsen zullen helpen bij het diagnosticeren van LNB. In dit hoofdstuk zijn zeven antistoftesten voor de diagnostiek van LNB geëvalueerd. Hierbij is gebruik gemaakt van een cross-sectionele studie-opzet, waarbij retrospectief alle opeenvolgende patiënten van de afdeling neurologie zijn geïncludeerd, waarvan een liquor-serum paar was ingestuurd naar het medisch microbiologisch laboratorium van het Diakonessenhuis gedurende een bepaalde tijdsperiode. Tevens is er voor elke antistoftest een multiparameter analyse uitgevoerd om te onderzoeken of de prestatiekarakteristieken van de antistoftest verbeterd konden worden door het meenemen van extra parameters. Deze extra parameters betroffen een aantal routinematige liquor parameters (pleiocytose, totaal eiwit, bloed-liquor barrière functionaliteit en intrathecale totale antistofsynthese), een *Borrelia*-specifieke (*Borrelia* species PCR) en een niet-specifieke liquor parameter (B-cel chemokine (C-X-C motief) ligand 13 [CXCL13]), en een *Borrelia*-specifieke serum parameter (*Borrelia*-specifieke antistoffen in het bloed). De resultaten van deze multiparameter analyses laten zien dat het gebruik van additionele parameters voor de diagnostiek van LNB in de meeste gevallen leidt tot een hogere sensitiviteit (variërend van 94,1% tot 100%) en een iets lagere specificiteit (variërend van 92,8% tot 96,4%) dan de sensitiviteit en specificiteit van de gevalideerde antistoftest zelf (variërend van 47,1% tot 100 % en van 95,7 % tot 100%, respectievelijk). De belangrijkste parameters die bijdragen aan een verbetering van de LNB diagnostiek zijn intrathecaal geproduceerde *Borrelia*-specifieke antistoffen, bloed-liquor barrière functionaliteit, intrathecale totale antistofsynthese, pleiocytose, CXCL13 in de liquor, en *Borrelia*-specifieke antistoffen in het bloed. Alhoewel meerdere studies de toegevoegde waarde van deze parameters voor de diagnostiek van LNB laten zien [8, 10-12], is onze studie de eerste die de relatieve bijdrage van deze parameters laat zien. Tevens toont onze studie aan welke parameters het meeste bijdragen en derhalve geschikt zijn om gebruikt te worden in een diagnostisch algoritme voor het aantonen van actieve LNB. Onze studie laat ook zien dat het meten van zowel de humorale als de cellulaire immuunrespons tegen een infectie met *B. burgdorferi* s.l. bijdraagt aan de diagnostiek van LNB, en dat individuele parameters (fout) negatief of (fout) positief kunnen zijn, maar toch een toegevoegde waarde hebben in breder verband. We zijn van mening dat deze resultaten dermate veelbelovend en belangrijk zijn dat een validatie met meerder centra nodig is. We stellen daarom voor om, in samenwerking met andere (inter)nationale laboratoria, een prospectieve studie op te zetten om te onderzoeken of een gestandaardiseerd diagnostisch algoritme ontwikkeld kan worden voor de diagnostiek van LNB gebaseerd op een multiparameter analyse.

De resultaten van de multiparameter analyses in **hoofdstuk 6** tonen aan dat van de onderzochte

parameters, de detectie van intrathecaal geproduceerde *Borrelia*-specifieke antistoffen het meeste bijdraagt aan de diagnostiek van LNB. Voor de detectie van pathogeen-specifieke antistoffen wordt veelal gebruik gemaakt van een enzym-linked immunosorbent assay (ELISA) [4]. ELISAs kunnen echter last hebben van een zogenaamd 'randeffect' [13]. Er is sprake van een randeffect als het resultaat van de metingen van eenzelfde antistofconcentratie in welletjes in de randen van de ELISA plaat hoger (of lager) zijn dan de metingen in welletjes in het midden van de ELISA plaat. Wanneer er sprake is van een randeffect in een ELISA plaat, kan dit gevolgen hebben voor de patiënten materialen die hierin getest worden. In **hoofdstuk 5** is aangetoond dat er sprake is van een randeffect bij gebruik van de commerciële Enzygnost Lyme link VlsE/IgG ELISA. Er is tevens aangetoond dat bij gebruik van deze ELISA voor de diagnostiek van LNB, de gevolgen van dit randeffect verminderd kunnen worden door een kleine aanpassing in het standaardprotocol van de producent. Dit aangepaste protocol werd vervolgens gebruikt om 149 liquor-serumparen te testen, afkomstig van de iets grotere monstercollectie gebruikt in **hoofdstuk 6** (en **4**). Middels simulatie werd aangetoond aan dat als het standaardprotocol van de producent zou zijn gebruikt, het randeffect voor deze studiepopulatie in het slechtste scenario had kunnen resulteren in 15 (10,1%) fout-positieve en twee (1,3%) fout-negatieve *Borrelia*-specifieke immunoglobuline (Ig)G antistof index resultaten. Het aangetoonde randeffect kan dus leiden tot foutieve LNB diagnostiek. De onderzoeksresultaten in **hoofdstuk 5** benadrukken het belang van een grondige validatie van ELISA testen voordat ze gebruikt worden voor routine diagnostiek, zoals bijvoorbeeld ook wordt vereist in de voorwaarden voor de ISO 15189-accreditatie [14].

Voor de diagnostiek van de meeste andere Lyme manifestaties wordt gebruik gemaakt van de detectie van *Borrelia*-specifieke antistoffen in het bloed, waarvoor een twee-staps teststrategie wordt geadviseerd [5, 15]. Deze twee-staps teststrategie heeft tot doel de diagnostische prestaties van antistoftesten te verbeteren door te starten met een test met een hoge sensitiviteit (d.w.z., een screening test) en dubieuze en positieve test uitslagen verkregen met de screening test te confirmeren met een tweede (confirmatie) test met een hoge specificiteit [7, 16, 17]. Vaak wordt voor de screening test gebruik gemaakt van een ELISA en voor de confirmatietest van een western blot (Noord-Amerika) of een immunoblot (Europa) [7]. Antistoftesten kunnen variëren in sensitiviteit, zoals is aangetoond in **hoofdstuk 6**, bijvoorbeeld doordat de antigenen in de test niet overeenkomen met de antigenen die tot expressie worden gebracht door de *Borrelia* bacterie die de infectie veroorzaakt. Deze mismatch kan veroorzaakt worden door de intra- en interspecies heterogeniteit van *B. burgdorferi* s.l [11, 18-22] en/of de antigene variatie van de infecterende stam tijdens het verloop van de infectie [23]. De sensitiviteit van een antistoftest kan ook beïnvloed worden door vroegtijdig antibiotica gebruik, wat de immuunrespons kan afbreken [24-28]. Dit antibiotica effect lijkt antistof-specifiek, aangezien antistoffen tegen het C6-peptide sneller afnemen dan antistoffen gericht tegen een cel lysaat van *B. burgdorferi* s.l [29, 30] of het p39-eiwit [29].

In **hoofdstuk 7** zijn twee screening testen (de C6 ELISA en de Serion ELISA) en twee twee-staps teststrategieën (confirmatie van dubieuze en positieve C6 ELISA en Serion ELISA resultaten met de *recom*Line immunoblot) voor het detecteren van *Borrelia*-specifieke antistoffen in bloed geëvalueerd. Het onderzoek in dit hoofdstuk toont aan dat antibiotische behandeling van een infectie met *B. burgdorferi* s.l sterk geassocieerd is met discordante screening test (ELISA) resultaten en twee-staps teststrategie (ELISA + immunoblot) resultaten (odds ratio [OR]: 10,52; $P < 0{,}001$ en OR: 9,98; $P = 0{,}014$, respectievelijk). Dit suggereert dat antibiotische behandeling van invloed is op het tempo waarin de antistoffen gericht tegen de verschillende antigenen in de ELISAs afnemen, waarbij antistoffen tegen het C6 peptide iets sneller lijken af te nemen dan die tegen cel lysaten van *B. burgdorferi* s.l., en bevestigt eerdere bevindingen [29, 30]. De discordante testresultaten in onze studie worden met name verklaard door de aanwezigheid van IgM tegen het buitenmembraan eiwit (outer surface protein) OspC, en in sommige gevallen ook tegen het

p41 flagel, zoals ook in eerdere studies is aangetoond [31, 32]. Deze resultaten onderschrijven de uitdagingen rondom de interpretatie van antistoftesten, waarmee men rekening dient te houden bij het gebruik van dergelijke testen voor de diagnostiek van de ziekte van Lyme.

In de vroege fase van een infectie zijn de antistoffen vaak nog niet aantoonbaar vanwege de nog opbouwende immuunrespons. Daarom zijn er nieuwe infectiemarkers nodig met een hoge sensitiviteit en specificiteit, met name in de beginfase van een infectie. In de afgelopen twee decennia zijn er steeds meer studies verschenen die de toegevoegde waarde laten zien van het aantonen van verhoogde concentraties van CXCL13 in de liquor van patiënten met een vroege LNB [33, 34]. De detectie van CXCL13 in de liquor is relatief eenvoudig in tegenstelling tot de vaak lastige berekeningen die nodig zijn voor het aantonen van de intrathecale synthese van *Borrelia*-specifieke antistoffen. In **hoofdstuk 4** zijn twee commerciële CXCL13-testen (de Quantikine CXCL13 ELISA en de *recom*Bead CXCL13 test) op liquor voor de diagnostiek van LNB geëvalueerd. Gebruikmakend van dezelfde studiepopulatie als in **hoofdstuk 6** (en bijna dezelfde studiepopulatie als die in **hoofdstuk 5**), is aangetoond dat het meten van CXCL13 in de liquor van toegevoegde waarde kan zijn voor het diagnosticeren van patiënten met een actieve LNB. Verder is aangetoond dat CXCL13 eerder in de liquor kan worden gedetecteerd dan intrathecaal geproduceerde *Borrelia*-specifieke antistoffen, en dat de bepaling van CXCL13 in de liquor met name van toegevoegde waarde is voor het aantonen van vroege LNB. De cutoff waardes voor beide testen verschillen echter wel, wat mogelijk verklaard kan worden door een verschil in methodiek [35]. Echter, in de literatuur worden ook verschillende cutoff waardes gerapporteerd bij gebruik van dezelfde testen [35-41]. Dit benadrukt de noodzaak voor verder onderzoek naar de oorzaak van deze verschillen en de mogelijkheden om een internationale referentiestandaard vast te stellen voor CXCL13 in de liquor.

De productie van CXCL13 in de liquor is onderdeel van de cellulaire immuunrespons. De cellulaire immuunrespons tegen *B. burgdorferi* s.l. wordt gekarakteriseerd door een sterke T helper (Th)1 respons, waarbij T cellen Th1-cytokines gaan produceren, waaronder interferon-gamma (IFN-γ). In de afgelopen jaren zijn er ook andere testen beschikbaar gekomen die zich richten op de cellulaire immuunrespons. Eén van deze testen is de enzym-linked immunospot (ELISpot) test waarmee *Borrelia*-specifieke IFN-γ producerende T cellen worden aangetoond. In **Hoofdstuk 2** en **3** zijn twee van dergelijke testen voor de diagnostiek van LNB geëvalueerd; een zelf ontwikkelde (zgn. 'in-house') *Borrelia* ELISpot test en de commerciële LymeSpot test. Veel Lymepatiënten gaan naar Duitsland waar commerciële IFN-γ ELISpot testen worden gebruikt, terwijl de klinische validatie van deze testen bij goed gedefinieerde patiëntengroepen ontbreekt. Echter, indien de IFN-γ ELISpot testuitslag bij deze patiënten positief is, wordt er vaak (langdurig) antibiotica gegeven voor de behandeling van actieve Lymeziekte [42]. Voor de evaluatie van de twee IFN-γ ELISpot testen in deze thesis werd gebruik gemaakt van een prospectieve 'case-control' studie, bestaande uit actieve LNB patiënten (cases), en drie controlegroepen bestaande uit behandelde LNB patiënten, en gezonde vrijwilligers met, en zonder een voorgeschiedenis van behandelde Lymeziekte (met name cutaan). De studies in **hoofdstuk 2** en **3** tonen aan dat de onderzochte IFN-γ ELISpot testen niet geschikt zijn om actieve LNB te diagnosticeren. De aanwezigheid van *Borrelia*-specifieke T-cel reactiviteit was vergelijkbaar tussen actieve LNB patiënten, behandelde LNB patiënten en behandelde gezonde vrijwilligers, en deze reactiviteit was hoger dan de *Borrelia*-specifieke T-cel reactiviteit bij onbehandelde gezonde vrijwilligers. De verhoogde *Borrelia*-specifieke T-cel reactiviteit bij beide behandelde groepen wordt waarschijnlijk verklaard door een eerdere, genezen ziekte van Lyme. De IFN-γ ELISpot test lijkt derhalve onderscheid te maken tussen *Borrelia*-naïeve en *Borrelia*-geïnfecteerde individuen.

CONCLUSIE

Wij zijn van mening dat het onderzoek in dit proefschrift heeft bijgedragen aan de doelen van dit proefschrift. De belangrijkste bevinding van dit proefschrift is het veelbelovende resultaat van het gebruik van meerdere parameters voor de diagnostiek van LNB (**hoofdstuk 6**), waarbij zowel de humorale als de cellulaire immuunrespons een belangrijke rol spelen (**hoofdstukken 4** en **6**). Het combineren van verschillende parameters in een diagnostisch algoritme, waarbij verschillende aspecten van het immuunsysteem in ogenschouw worden genomen, geeft concrete en direct uitvoerbare handvatten om een duidelijker onderscheid maken tussen een actieve infectie en een eerdere doorgemaakte infectie en zal de diagnostiek van LNB verbeteren. We zijn ook van mening dat de ontwikkeling van een diagnostisch algoritme van toegevoegde waarde kan zijn voor de diagnostiek van andere Lyme manifestaties.

De variatie in de onderzochte ELISA in **hoofdstuk 5** benadrukt het belang van het uitvoeren van een gedegen validatie van diagnostische testen alvorens deze worden gebruikt in de routinediagnostiek. Als alle laboratoria dit doen, en dus goed inzicht hebben in de (on) mogelijkheden van de test die zij gebruiken, zal de diagnostiek van de ziekte van Lyme verbeteren. In **hoofdstuk 7** is aangetoond dat antibioticagebruik invloed heeft op de humorale immuunrespons. Dit kan eventuele verschillen in de resultaten tussen verschillende antistoftesten verklaren en dit zal zowel de laboratorium-specialisten als de artsen helpen bij het interpreteren van de resultaten van antistoftesten.

Tot slot geeft de gedegen evaluatie van twee IFN-γ ELISpot testen (**hoofdstukken 2** en **3**) inzicht in de beperkingen van deze testen om actieve LNB aan te tonen en dit is van belang voor artsen én patiënten. Hopelijk zal dit het onnodig antibioticagebruik op basis van onterechte diagnoses, gesteld op basis van IFN-γ ELISpot testuitslagen, beperken. De resultaten van beide studies bieden ook een basis waarmee artsen in gesprek kunnen gaan met patiënten in het geval dat patiënten elders met soortgelijke testen gediagnosticeerd zijn.

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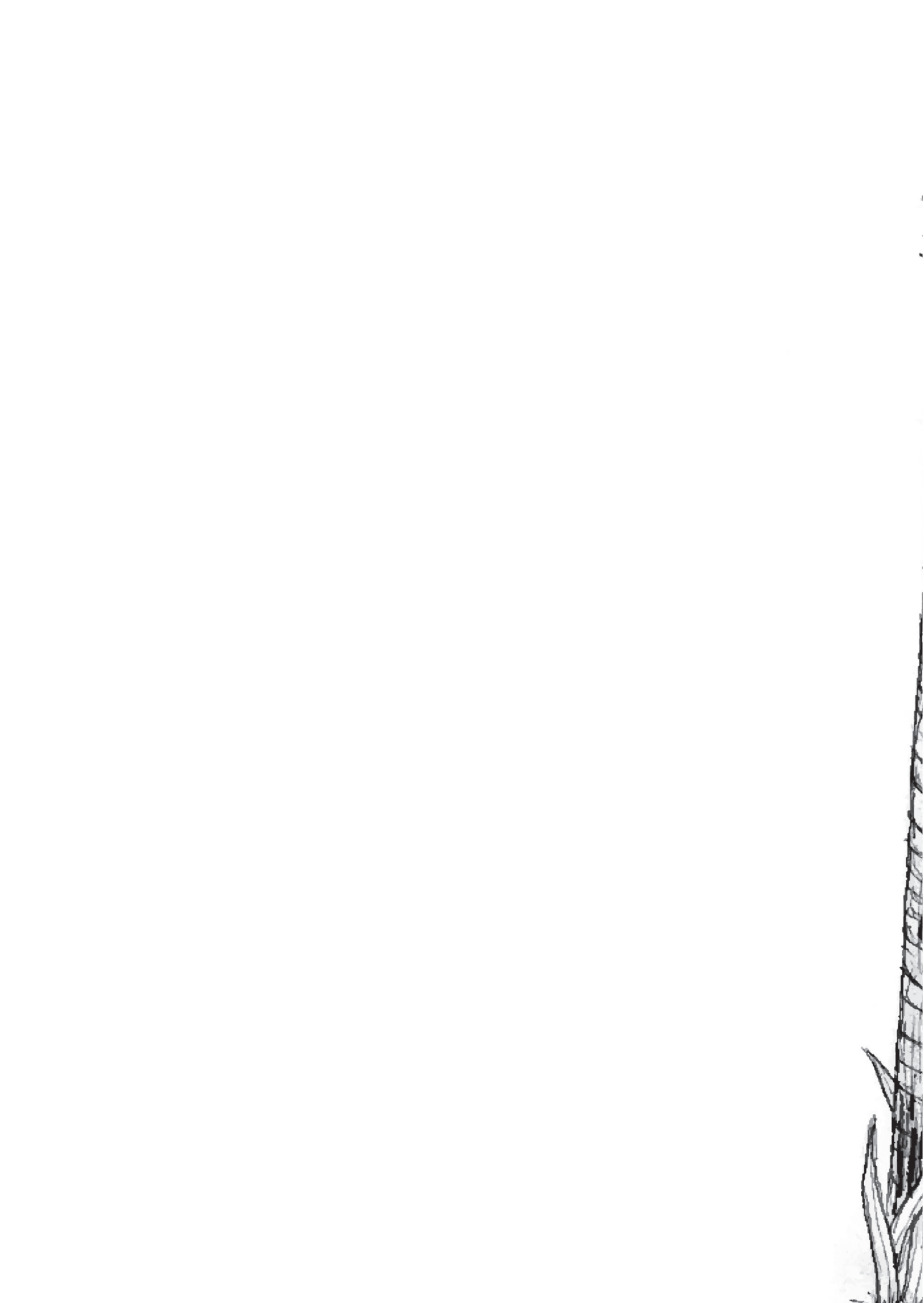

APPENDICES

CURRICULUM VITAE

Tamara van Gorkom werd op 13 september 1975 geboren in Deventer. Ze groeide op in Leusden, waar zij het basisonderwijs volgde op basisschool de Vallei. In 1993 haalde ze haar VWO-diploma aan het Corderius College te Amersfoort en startte zij haar opleiding aan de Hogeschool van Utrecht op de faculteit Natuur en Techniek, sector Laboratoriumonderzoek en Chemische Technologie. Na een diagnostiekstage bij de afdeling Medische Microbiologie van Ziekenhuis Eemland (locatie St. Elisabeth) in Amersfoort en een onderzoekstage op de afdeling parasitologie van het Microbiologische Laboratorium voor de Gezondheidsbescherming (MGB) van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) in Bilthoven, behaalde zij in 1997 haar diploma in de afstudeerrichting Medische Microbiologie. In hetzelfde jaar startte ze haar loopbaan op het Laboratorium voor Infectieziekten Onderzoek (LIO) van het RIVM, alwaar zij de spoligotyping op *Mycobacterium tuberculosis* complex stammen uitvoerde. Tevens deed ze onderzoek naar de oorsprong van DNA polymorfismen in het Direct Repeat Locus van *M. tuberculosis* complex stammen. Ze vervolgde haar loopbaan op de Stanford Universiteit in Palo Alto, Californië, Verenigde Staten, alwaar ze onderzoek deed naar de ontwikkeling van een high-throughput membraan-gebaseerde hybridisatiemethode om de fylogenetische relatie tussen verschillende *M. tuberculosis* complex stammen te kunnen bestuderen. Na een jaar keerde ze terug naar Nederland en vervolgde ze haar loopbaan op het Medisch Microbiologisch Laboratorium van het Meander Medisch Centrum in Amersfoort. Vervolgens keerde Tamara terug naar het RIVM, ditmaal op het Laboratorium voor Infectieziekten diagnostiek en Screening (LIS), alwaar ze onderzoek deed naar de genetische verschillen in *Bordetella pertussis* stammen in Nederland met behulp van micro-arrays. In 2008 startte Tamara haar werkzaamheden op de afdeling Medische Microbiologie en Immunologie (MMI) van het Diakonessenhuis in Utrecht en is ze in 2012 gestart met het Lyme onderzoek. In 2015 is een samenwerking gestart met het RIVM op het gebied van de ziekte van Lyme. Dit was tevens het startsein van haar promotietraject en het moment dat ze naast haar baan op het MMI van het Diakonessenhuis weer is gaan werken op het RIVM, op de afdeling Bacteriële en Parasitologische Diagnostiek (BPD). Sinds september 2022 is Tamara volledig in dienst van het Diakonessenhuis Utrecht alwaar zij als post-doc verder onderzoek gaat doen naar de ziekte van Lyme. Daarnaast is ze als gast verbonden aan het RIVM, afdeling BPD.

DANKWOORD

Er zijn veel mensen die hebben bijgedragen aan dit proefschrift die ik graag wil bedanken. Allereerst wil ik de patiënten en vrijwilligers bedanken voor hun deelname aan het Lyme-onderzoek. Zonder hun deelname was het onderzoek in dit proefschrift niet mogelijk geweest. Daarnaast wil ik iedereen bedanken met wie ik de afgelopen jaren heb samengewerkt. De volgende personen wil ik in het bijzonder bedanken:

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Mijn co-promotoren Kristin en Steven. Kristin, wij kennen elkaar al ruim 25 jaar en tijdens onze tb-tijd had ik nooit gedacht dat we samen dit Lyme avontuur aan zouden gaan. Naast dat ik de afgelopen jaren ontzettend veel van jou geleerd heb, was je ook een fijne kamergenoot en stond je voor me klaar met een luisterend oor en goede raad. Steven, jij hebt me gestimuleerd om voor de research te gaan en zag in mij wat ik destijds zelf (nog) niet zag. Jouw enthousiasme, positieve energie, passie voor de wetenschap en niet aflatende vertrouwen in mij is ontzettend stimulerend en ik kijk dan ook met veel plezier naar de toekomst, waarin we - samen met anderen - nog heel veel mooie onderzoeken gaan doen.

Willem en Sanjay. Willem, als neuroloog ben jij vanaf de start betrokken geweest bij het Lyme-onderzoek. Bedankt voor alle inclusies vanuit de neurologie en de prettige samenwerking. Hopelijk gaan we nog heel veel meer onderzoek doen. Sanjay, als internist infectieziekten ben ook jij vanaf de start betrokken bij het Lyme-onderzoek. Bedankt voor jouw inzet, met name met betrekking tot de inclusie van de vrijwilligers, maar ook voor jouw actieve bijdrage aan de kwartaal overleggen.

Michiel, jij bent als medisch immunoloog bij het Lyme-onderzoek betrokken geraakt. Fijn dat je deel uitmaakt van het team en bedankt voor het sparren.

De collega's en stagiaires van de afdelingen MMI en KCHI van het Diakonessenhuis Utrecht en de collega's en stagiaires van het RIVM die op enigerlei wijze hebben bijgedragen aan het onderzoek. In de naastgelegen figuur wil ik jullie op mijn eigen manier erkennen.

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Beste Chiel, Jaap, Louk en Barbara. Via Elisabeth ben ik in een warm bad terecht gekomen. Bedankt voor jullie aanmoedigingen en enthousiasme. Chiel, je bent er helaas niet meer bij, maar in gedachten zeker wel.

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