Universiteit
Leiden
The Netherlands

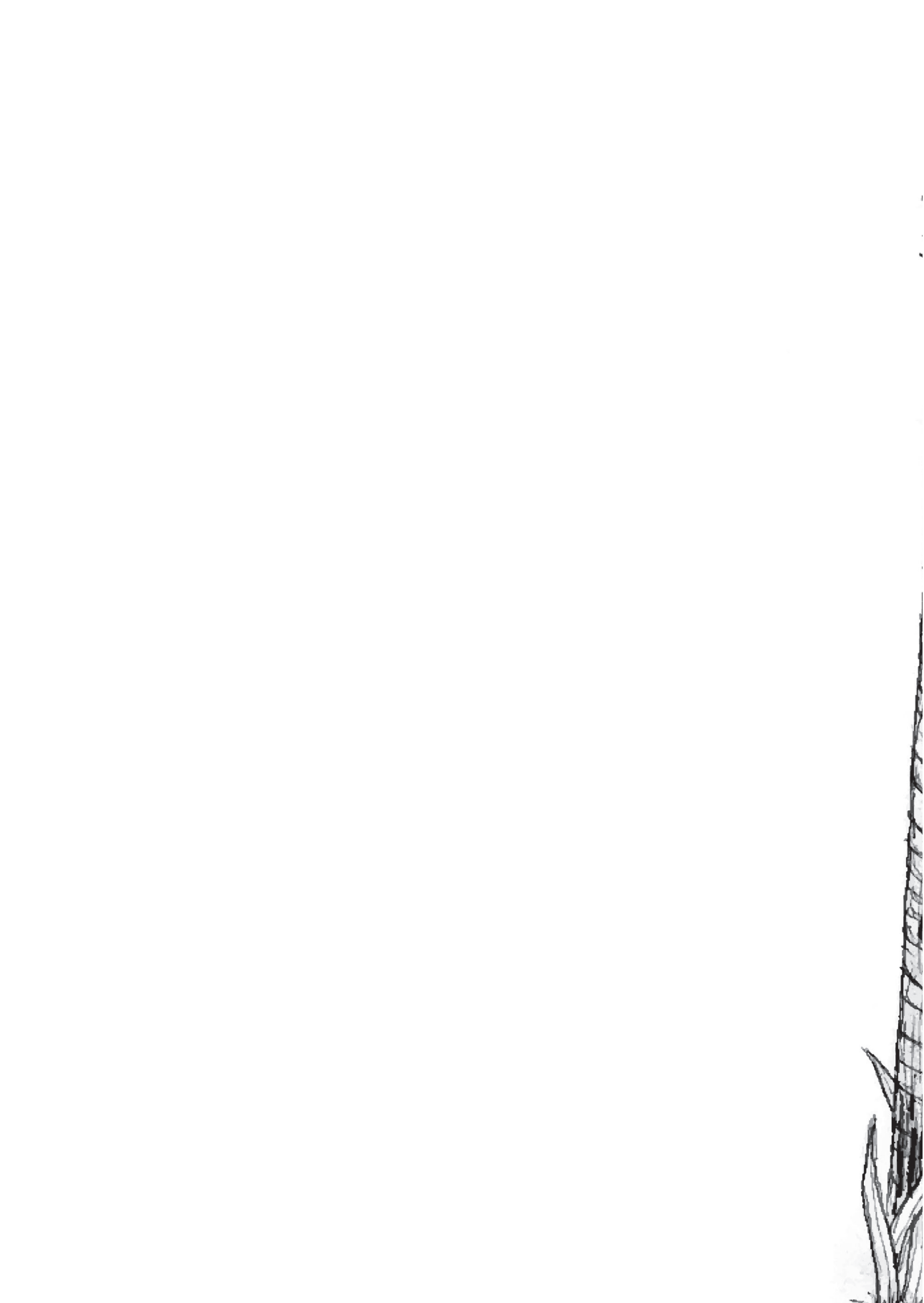
Immunodiagnostics of Lyme neuroborreliosis

Gorkom, T. van

Citation
Gorkom, T. van. (2022, December 8). *Immunodiagnostics of Lyme neuroborreliosis*. Retrieved from https://hdl.handle.net/1887/3494393

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/3494393

Note: To cite this publication please use the final published version (if applicable).



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.

REFERENCES

1. Stanek G, Fingerle V, Hunfeld KP, Jaulhac B, Kaiser R, Krause A, Kristoferitsch W, O'Connell S, Ornstein K, Strle F, Gray J. 2011. Lyme borreliosis: clinical case definitions for diagnosis and management in Europe. Clin Microbiol Infect 17:69-79. doi: 10.1111/j.1469-0691.2010.03175.x.
2. Eldin C, Raffetin A, Bouiller K, Hansmann Y, Roblot F, Raoult D, Parola P. 2019. Review of European and American guidelines for the diagnosis of Lyme borreliosis. Med Mal Infect 49:121-132. doi: 10.1016/j.medmal.2018.11.011.
3. Talagrand-Reboul E, Raffetin A, Zachary P, Jaulhac B, Eldin C. 2020. Immunoserological Diagnosis of Human Borrelioses: Current Knowledge and Perspectives. Front Cell Infect Microbiol 10:241. doi: 10.3389/fcimb.2020.00241.
4. Leeflang MMG, Ang CW, Berkhout J, Bijlmer HA, Van BW, Brandenburg AH, van Burgel ND, van Dam AP, Dessau RB, Fingerle V, Hovius JWR, Jaulhac B, Meijer B, Van PW, Schellekens JFP, Spijker R, Stelma FF, Stanek G, Verduyn-Lunel F, Zeller H, Sprong H. 2016. The diagnostic accuracy of serological tests for Lyme borreliosis in Europe: a systematic review and meta-analysis. BMC Infect Dis 16. doi: 10.1186/s12879-016-1468-4.
5. Spellberg B, Edwards JE, Jr. 2001. Type 1/Type 2 immunity in infectious diseases. Clin Infect Dis 32:76-102. doi: 10.1086/317537.
6. Mygland A, Ljostad U, Fingerle V, Rupprecht T, Schmutzhard E, Steiner I. 2010. EFNS guidelines on the diagnosis and management of European Lyme neuroborreliosis. Eur J Neurol 17:8-4. doi: 10.1111/j.1468-1331.2009.02862.x.
7. Reiber H, Peter JB. 2001. Cerebrospinal fluid analysis: disease-related data patterns and evaluation programs. J Neurol Sci 184:101-22. doi: 10.1016/s0022-510x(00)00501-3.
8. Djukic M, Schmidt-Samoa C, Lange P, Spreer A, Neubieser K, Eiffert H, Nau R, Schmidt H. 2012. Cerebrospinal fluid findings in adults with acute Lyme neuroborreliosis. J Neurol 259:630-6. doi: 10.1007/s00415-011-6221-8.
9. Hansen K, Crone C, Kristoferitsch W. 2013. Lyme neuroborreliosis. Handb Clin Neurol 115:559-75. doi: 10.1016/B978-0-444-52902-2.00032-1.
10. Rupprecht TA, Manz KM, Fingerle V, Lechner C, Klein M, Pfirrmann M, Koedel U. 2018. Diagnostic value of cerebrospinal fluid CXCL13 for acute Lyme neuroborreliosis. A systematic review and meta-analysis. Clin Microbiol Infect 24:1234-1240. doi: 10.1016/j.cmi.2018.04.007.
11. Hofhuis A, Harms M, Bennema S, van den Wijngaard CC, van Pelt W. 2015. Physician reported incidence of early and late Lyme borreliosis. Parasit Vectors 8:161. doi: 10.1186/s13071-015-0777-6.
12. Reiber H. 1995. External quality assessment in clinical neurochemistry: survey of analysis for cerebrospinal fluid (CSF) proteins based on CSF/serum quotients. Clin Chem 41:256-63. doi: 10.1093/clinchem/41.2.256.
13. Liaw A, Wiener M. 2002. Classification and regression by randomForest. R news 2:18-22. https://CRAN.R-project.org/doc/Rnews/.
14. Burt SM, Carter TJ, Kricka LJ. 1979. Thermal characteristics of microtitre plates used in immunological assays. J Immunol Methods 31:231-6. doi: 10.1016/0022-1759(79)90135-2.
15. Oliver DG, Sanders AH, Hogg RD, Hellman JW. 1981. Thermal gradients in microtitration plates. Effects on enzyme-linked immunoassay. J Immunol Methods 42:195-201. doi: 10.1016/0022-1759(81)90149-6.
16. Auld DS, Coassin PA, Coussens NP, Hensley P, Klumpp-Thomas C, Michael S, Sitta Sittampalam G, Joseph Trask O, Wagner BK, Weidner JR, Wildey M, Dahlin JL. 2020. Microplate Selection and Recommended Practices in High-throughput Screening and Quantitative Biology. 2020 Jun 1. *In*: Markossian S, Sittampalam GS, Grossman A, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the

National Center for Advancing Translational Sciences; 2004-. Available from: https://www.ncbi.nlm.nih.gov/sites/books/NBK558077/. Retrieved 22 October 2020. The Assay Guidance Manual.
17. Lilyanna S, Ng EMW, Moriguchi S, Chan SP, Kokawa R, Huynh SH, Chong PCJ, Ng YX, Richards AM, Ng TW, Liew OW. 2018. Variability in Microplate Surface Properties and Its Impact on ELISA. J Appl Lab Med 2:687-699. doi: 10.1373/jalm.2017.023952.
18. Shekarchi IC, Sever JL, Lee YJ, Castellano G, Madden DL. 1984. Evaluation of various plastic microtiter plates with measles, toxoplasma, and gamma globulin antigens in enzyme-linked immunosorbent assays. J Clin Microbiol 19:89-96. doi: 10.1128/JCM.19.2.89-96.1984.
19. Faruq A, Dadson L, Cox H, Alcock F, Parker AR. 2010. Technical and diagnostic performance of five commercial anti-diphtheria toxoid IgG enzyme-linked immunosorbent assay kits. Clin Vaccine Immunol 17:1612-6. doi: 10.1128/CVI.00077-10.
20. Graham DA, Mawhinney KA, McShane J, Connor TJ, Adair BM, Merza M. 1997. Standardization of enzyme-linked immunosorbent assays (ELISAs) for quantitative estimation of antibodies specific for infectious bovine rhinotracheitis virus, respiratory syncytial virus, parainfluenza-3 virus, and bovine viral diarrhea virus. J Vet Diagn Invest 9:24-31. doi: 10.1177/104063879700900105.
21. International Organization for Standardization. 2012. ISO 15189:2012 Medical laboratories — Requirements for quality and competence. https://www.iso.org/standard/56115.html.
22. Waddell LA, Greig J, Mascarenhas M, Harding S, Lindsay R, Ogden N. 2016. The Accuracy of Diagnostic Tests for Lyme Disease in Humans, A Systematic Review and Meta-Analysis of North American Research. PLoS One 11:e0168613. doi: 10.1371/journal.pone.0168613.
23. Roessler D, Hauser U, Wilske B. 1997. Heterogeneity of BmpA (P39) among European isolates of *Borrelia burgdorferi* sensu lato and influence of interspecies variability on serodiagnosis. Journal of clinical microbiology 35:2752-2758. doi: 10.1128/jcm.35.11.2752-2758.1997.
24. Wang G, van Dam AP, Schwartz I, Dankert J. 1999. Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. Clin Microbiol Rev 12:633-53. doi: 10.1128/CMR.12.4.633.
25. Ornstein K, Berglund J, Bergstrom S, Norrby R, Barbour AG. 2002. Three major Lyme *Borrelia* genospecies (*Borrelia burgdorferi* sensu stricto, *B. afzelii* and *B. garinii*) identified by PCR in cerebrospinal fluid from patients with neuroborreliosis in Sweden. Scandinavian journal of infectious diseases 34:341-346. doi: 10.1080/00365540110080313.
26. Margos G, Vollmer SA, Ogden NH, Fish D. 2011. Population genetics, taxonomy, phylogeny and evolution of *Borrelia burgdorferi* sensu lato. Infect Genet Evol 11:1545-63. doi: 10.1016/j.meegid.2011.07.022.
27. Brisson D, Baxamusa N, Schwartz I, Wormser GP. 2011. Biodiversity of *Borrelia burgdorferi* strains in tissues of Lyme disease patients. PLoS One 6:e22926. doi: 10.1371/journal.pone.0022926.
28. Zhang JR, Hardham JM, Barbour AG, Norris SJ. 1997. Antigenic variation in Lyme disease *borreliae* by promiscuous recombination of VMP-like sequence cassettes. Cell 89:275-85. doi: 10.1016/s0092-8674(00)80206-8.
29. Dattwyler RJ, Volkman DJ, Luft BJ, Halperin JJ, Thomas J, Golightly MG. 1988. Seronegative Lyme disease. Dissociation of specific T- and B-lymphocyte responses to *Borrelia burgdorferi*. N Engl J Med 319:1441-1446. doi: 10.1056/NEJM198812013192203.
30. Baig S, Olsson T, Hansen K, Link H. 1991. Anti-*Borrelia burgdorferi* antibody response over the course of Lyme neuroborreliosis. Infect Immun 59:1050-6. doi: 10.1128/iai.59.3.1050-1056.1991.

31. Ledue TB, Collins MF, Craig WY. 1996. New laboratory guidelines for serologic diagnosis of Lyme disease: evaluation of the two-test protocol. J Clin Microbiol 34:2343-50. doi: 10.1128/jcm.34.10.2343-2350.1996.
32. Branda JA, Steere AC. 2021. Laboratory Diagnosis of Lyme Borreliosis. Clin Microbiol Rev 34. doi: 10.1128/CMR.00018-19.
33. Rebman AW, Crowder LA, Kirkpatrick A, Aucott JN. 2015. Characteristics of seroconversion and implications for diagnosis of post-treatment Lyme disease syndrome: acute and convalescent serology among a prospective cohort of early Lyme disease patients. Clinical rheumatology 34:585-589. doi: 10.1007/s10067-014-2706-z.
34. Philipp MT, Bowers LC, Fawcett PT, Jacobs MB, Liang FT, Marques AR, Mitchell PD, Purcell JE, Ratterree MS, Straubinger RK. 2001. Antibody response to IR6, a conserved immunodominant region of the VlsE lipoprotein, wanes rapidly after antibiotic treatment of *Borrelia burgdorferi* infection in experimental animals and in humans. J Infect Dis 184:870-878. doi: 10.1086/323392.
35. Peltomaa M, McHugh G, Steere AC. 2003. Persistence of the antibody response to the VlsE sixth invariant region (IR6) peptide of *Borrelia burgdorferi* after successful antibiotic treatment of Lyme disease. The Journal of infectious diseases 187:1178-1186. doi: 10.1086/374376.
36. Aguero-Rosenfeld ME, Nowakowski J, Bittker S, Cooper D, Nadelman RB, Wormser GP. 1996. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. J Clin Microbiol 34:1-9. doi: 10.1128/jcm.34.1.1-9.1996.
37. Kalish RA, McHugh G, Granquist J, Shea B, Ruthazer R, Steere AC. 2001. Persistence of immunoglobulin M or immunoglobulin G antibody responses to *Borrelia burgdorferi* 10-20 years after active Lyme disease. Clin Infect Dis 33:780-785. doi: 10.1086/322669.
38. Ljostad U, Mygland A. 2008. CSF B-lymphocyte chemoattractant (CXCL13) in the early diagnosis of acute Lyme neuroborreliosis. J Neurol 255:782. doi: 10.1007/s00415-008-0974-8.
39. Rupprecht TA, Pfister HW, Angele B, Kastenbauer S, Wilske B, Koedel U. 2005. The chemokine CXCL13 (BLC): a putative diagnostic marker for neuroborreliosis. Neurology 65:448-50. doi: 10.1212/01.wnl.0000171349.06645.79.
40. Senel M, Rupprecht TA, Tumani H, Pfister HW, Ludolph AC, Brettschneider J. 2010. The chemokine CXCL13 in acute neuroborreliosis. J Neurol Neurosurg Psychiatry 81:929-33. doi: 10.1136/jnnp.2009.195438.
41. Markowicz M, Schotta AM, Kundi M, Bogovic P, Ogrinc K, Strle F, Stanek G. 2018. CXCL13 concentrations in cerebrospinal fluid of patients with Lyme neuroborreliosis and other neurological disorders determined by Luminex and ELISA. Ticks Tick Borne Dis 9:1137-1142. doi: 10.1016/j.ttbdis.2018.04.008.
42. Henningsson AJ, Gyllemark P, Lager M, Skogman BH, Tjernberg I. 2016. Evaluation of two assays for CXCL13 analysis in cerebrospinal fluid for laboratory diagnosis of Lyme neuroborreliosis. Apmis 124:985-990. doi: 10.1111/apm.12596.
43. Tjernberg I, Henningsson AJ, Eliasson I, Forsberg P, Ernerudh J. 2011. Diagnostic performance of cerebrospinal fluid chemokine CXCL13 and antibodies to the C6-peptide in Lyme neuroborreliosis. J Infect 62:149-58. doi: 10.1016/j.jinf.2010.11.005.
44. Hytonen J, Kortela E, Waris M, Puustinen J, Salo J, Oksi J. 2014. CXCL13 and neopterin concentrations in cerebrospinal fluid of patients with Lyme neuroborreliosis and other diseases that cause neuroinflammation. J Neuroinflammation 11:103. doi: 10.1186/1742-2094-11-103.
45. Schmidt C, Plate A, Angele B, Pfister HW, Wick M, Koedel U, Rupprecht TA. 2011. A prospective study on the role of CXCL13 in Lyme neuroborreliosis. Neurology 76:1051-8. doi: 10.1212/WNL.0b013e318211c39a.

46. van Burgel ND, Bakels F, Kroes AC, van Dam AP. 2011. Discriminating Lyme neuroborreliosis from other neuroinflammatory diseases by levels of CXCL13 in cerebrospinal fluid. J Clin Microbiol 49:2027-30. doi: 10.1128/JCM.00084-11.
47. Barstad B, Tveitnes D, Noraas S, Selvik Ask I, Saeed M, Bosse F, Vigemyr G, Huber I, Oymar K. 2017. Cerebrospinal fluid B-lymphocyte chemoattractant CXCL13 in the diagnosis of acute Lyme neuroborreliosis in children. Pediatr Infect Dis J 36:e286-e292. doi: 10.1097/INF.0000000000001669.
48. Strle K, Jones KL, Drouin EE, Li X, Steere AC. 2011. *Borrelia burgdorferi* RST1 (OspC type A) genotype is associated with greater inflammation and more severe Lyme disease. Am J Pathol 178:2726-39. doi: 10.1016/j.ajpath.2011.02.018.
49. Strle K, Shin JJ, Glickstein LJ, Steere AC. 2012. Association of a Toll-like receptor 1 polymorphism with heightened Th1 inflammatory responses and antibiotic-refractory Lyme arthritis. Arthritis Rheum 64:1497-507. doi: 10.1002/art.34383.
50. Picha D, Moravcova L, Smiskova D. 2016. Prospective study on the chemokine CXCL13 in neuroborreliosis and other aseptic neuroinfections. J Neurol Sci 368:214-20. doi: 10.1016/j.jns.2016.05.059.
51. Tumani H, Nolker G, Reiber H. 1995. Relevance of cerebrospinal fluid variables for early diagnosis of neuroborreliosis. Neurology 45:1663-70. doi: 10.1212/wnl.45.9.1663.
52. Ljostad U, Skarpaas T, Mygland A. 2007. Clinical usefulness of intrathecal antibody testing in acute Lyme neuroborreliosis. Eur J Neurol 14:873-6. doi: 10.1111/j.1468-1331.2007.01799.x.
53. Hansen K, Lebech AM. 1991. Lyme neuroborreliosis: a new sensitive diagnostic assay for intrathecal synthesis of *Borrelia burgdorferi*-specific immunoglobulin G, A, and M. Ann Neurol 30:197-205. doi: 10.1002/ana.410300212.
54. Gubanova K, Lang J, Latzko J, Novotna B, Perneczky J, Pingitzer S, Purer P, Wuchty B, Waiß C, Sellner J. 2021. Peripheral neuropathy due to neuroborreliosis: Insensitivity for CXCL13 as early diagnostic marker. International Journal of Infectious Diseases 105:460-462.
55. Ogrinc K, Hernandez SA, Korva M, Bogovic P, Rojko T, Lusa L, Chiumento G, Strle F, Strle K. 2022. Unique Clinical, Immune, and Genetic Signature in Patients with Borrelial Meningoradiculoneuritis(1). Emerg Infect Dis 28. doi: 10.3201/eid2804.211831.
56. Wutte N, Berghold A, Loffler S, Zenz W, Daghofer E, Krainberger I, Kleinert G, Aberer E. 2011. CXCL13 chemokine in pediatric and adult neuroborreliosis. Acta Neurol Scand 124:321-8. doi: 10.1111/j.1600-0404.2010.01477.x.
57. Wagner JN, Weis S, Kubasta C, Panholzer J, von Oertzen TJ. 2018. CXCL13 as a diagnostic marker of neuroborreliosis and other neuroinflammatory disorders in an unselected group of patients. J Neurol 265:74-81. doi: 10.1007/s00415-017-8669-7.
58. Ziegler K, Rath A, Schoerner C, Meyer R, Bertsch T, Erbguth F, Bogdan C, Steinmann J, Held J. 2020. Comparative analysis of the Euroimmun CXCL13 enzyme-linked immunosorbent assay and the ReaScan lateral flow immunoassay for diagnosis of Lyme neuroborreliosis. J Clin Microbiol 58. doi: 10.1128/JCM.00207-20.
59. Krumbholz M, Theil D, Cepok S, Hemmer B, Kivisakk P, Ransohoff RM, Hofbauer M, Farina C, Derfuss T, Hartle C, Newcombe J, Hohlfeld R, Meinl E. 2006. Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. Brain 129:200-11. doi: 10.1093/brain/awh680.
60. Sellebjerg F, Bornsen L, Khademi M, Krakauer M, Olsson T, Frederiksen JL, Sorensen PS. 2009. Increased cerebrospinal fluid concentrations of the chemokine CXCL13 in active MS. Neurology 73:2003-10. doi: 10.1212/WNL.0b013e3181c5b457.
61. Fischer L, Korfel A, Pfeiffer S, Kiewe P, Volk HD, Cakiroglu H, Widmann T, Thiel E. 2009. CXCL13 and CXCL12 in central nervous system lymphoma patients. Clin Cancer Res 15:5968-73. doi: 10.1158/1078-0432.CCR-09-0108.
62. Yu Q, Cheng Y, Wang Y, Wang C, Lu H, Guan Z, Huang J, Gong W, Shi M, Ni L, Wu J, Peng R,

Zhou P. 2017. Aberrant humoral immune responses in neurosyphilis: CXCL13/CXCR5 play a pivotal role for B-cell recruitment to the cerebrospinal fluid. J Infect Dis 216:534-544. doi: 10.1093/infdis/jix233.
63. Dersch R, Hottenrott T, Senel M, Lehmensiek V, Tumani H, Rauer S, Stich O. 2015. The chemokine CXCL13 is elevated in the cerebrospinal fluid of patients with neurosyphilis. Fluids Barriers CNS 12:12. doi: 10.1186/s12987-015-0008-8.
64. Pai M, Behr MA, Dowdy D, Dheda K, Divangahi M, Boehme CC, Ginsberg A, Swaminathan S, Spigelman M, Getahun H, Menzies D, Raviglione M. 2016. Tuberculosis. Nature Reviews Disease Primers 2:16076. doi: 10.1038/nrdp.2016.76.
65. Kak G, Raza M, Tiwari BK. 2018. Interferon-gamma (IFN-gamma): Exploring its implications in infectious diseases. Biomol Concepts 9:64-79. doi: 10.1515/bmc-2018-0007.
66. Chesler DA, Reiss CS. 2002. The role of IFN-γ in immune responses to viral infections of the central nervous system. Cytokine & Growth Factor Reviews 13:441-454. doi: 10.1016/S1359-6101(02)00044-8.
67. Mak WA, Koeleman JGM, Ong DSY. 2022. Development of an in-house SARS-CoV-2 interferon-gamma ELISpot and plate reader-free spot detection method. J Virol Methods 300:114398. doi: 10.1016/j.jviromet.2021.114398.
68. Kruse M, Dark C, Aspden M, Cochrane D, Competiello R, Peltz M, Torres L, Wrighton-Smith P, Dudek M. 2021. Performance of the T-SPOT®.COVID test for detecting SARS-CoV-2-responsive T cells. International Journal of Infectious Diseases 113:155 - 161. doi: 10.1016/j.ijid.2021.09.073
69. Thijsen S, Heron M, Gremmels H, van der Kieft R, Reusken C, Kremer K, Limonard G, Bossink A. 2020. Elevated nucleoprotein-induced interferon-γ release in COVID-19 patients detected in a SARS-CoV-2 enzyme-linked immunosorbent spot assay. J Infect 81:452-482. doi: 10.1016/j.jinf.2020.06.015.
70. Barabas S, Spindler T, Kiener R, Tonar C, Lugner T, Batzilla J, Bendfeldt H, Rascle A, Asbach B, Wagner R, Deml L. 2017. An optimized IFN-γ ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. BMC Immunol 18:14. doi: 10.1186/s12865-017-0195-y.
71. Forner G, Saldan A, Mengoli C, Gussetti N, Palù G, Abate D. 2016. Cytomegalovirus (CMV) Enzyme-Linked Immunosorbent Spot Assay but Not CMV QuantiFERON Assay Is a Novel Biomarker To Determine Risk of Congenital CMV Infection in Pregnant Women. J Clin Microbiol 54:2149-54. doi: 10.1128/jcm.00561-16.
72. Smith JG, Liu X, Kaufhold RM, Clair J, Caulfield MJ. 2001. Development and validation of a gamma interferon ELISPOT assay for quantitation of cellular immune responses to varicella-zoster virus. Clin Diagn Lab Immunol 8:871-9. doi: 10.1128/cdli.8.5.871-879.2001.
73. Ryan JE, Ovsyannikova IG, Poland GA. 2005. Detection of Measles Virus-Specific Interferon-γ-Secreting T-Cells by ELISPOT, p 207-217. *In* Kalyuzhny AE (ed), Handbook of ELISPOT: Methods and Protocols doi:doi: 10.1385/1-59259-903-6:207. Humana Press, Totowa, NJ.
74. Smith JG, Levin M, Vessey R, Chan IS, Hayward AR, Liu X, Kaufhold RM, Clair J, Chalikonda I, Chan C, Bernard M, Wang WW, Keller P, Caulfield MJ. 2003. Measurement of cell-mediated immunity with a Varicella-Zoster Virus-specific interferon-gamma ELISPOT assay: responses in an elderly population receiving a booster immunization. J Med Virol 70 Suppl 1:S38-41. doi: 10.1002/jmv.10318.
75. Gong W, Wu X. 2021. Differential Diagnosis of Latent Tuberculosis Infection and Active Tuberculosis: A Key to a Successful Tuberculosis Control Strategy. Front Microbiol 12:745592. doi: 10.3389/fmicb.2021.745592.
76. Arminlabs. *Borrelia* ELISpot (T-Cell-Spot/IGRA: Interferon-Gamma-Release Assay). Available at: https://www.arminlabs.com/en/tests/elispot. Accessed November 2018.

77. BCA-clinic. Is the LymeSpot revised more effective than the traditional ELISpot. 2018. Available at: https://www.bca-clinic.de/en/is-the-lymespot-revised-more-effective-than-the-traditional-elispot/. Accessed February 2019.
78. Nordic Laboratories. *Borrelia* Elispot LTT (previously named Lyme Elispot LTT). http://www.nordiclabs.com/EDetail.aspx?id=2913. Accessed 18 June 2018 and 28 February 2022.
79. Regenerus Labs. Partnering with BCA Labs - The Viral and Lyme Experts. The EliSpot (Interferon-Gamma-Test) and the "LymeSpot Revised": The new EliSpot. https://regeneruslabs.com/blogs/blog/partnering-with-bca-labs-the-viral-and-lyme-experts?_pos=1&_sid=afd85ce7f&_ss=r. Accessed 18 June 2018 and 28 February 2022.
80. Infectolab Americas. *Borrelia burgdorferi* ELISPOT. https://www.infectolab-americas.com/*borrelia-burgdorferi*. Accessed 28 February 2022.
81. IMD Labor Berlin. The lymphocyte transformation test LTT Lyme Disease. https://www.imd-berlin.de/en/subject-information/diagnostics-information/lyme-disease-clinical-symptoms-and-diagnostics. Accessed 18 June 2018 and 28 February 2022.
82. Alstrup K, Bech M. 2016. TV2_Documentar. [Snyd eller borrelia]. Available at: https://www.dailymotion.com/video/x4wmdyj. Accessed February 2019.
83. Coleman AS, Pal U. 2009. BBK07, a dominant in vivo antigen of *Borrelia burgdorferi*, is a potential marker for serodiagnosis of Lyme disease. Clinical and Vaccine Immunology 16:1569-1575. doi: 10.1128/CVI.00301-09.
84. Ramamoorthy R, Philipp MT. 1998. Differential expression of *Borrelia burgdorferi* proteins during growth in vitro. Infect Immun 66:5119-24. doi: 10.1128/IAI.66.11.5119-5124.1998.
85. Baranton G, Postic D, Saint G, I, Boerlin P, Piffaretti JC, Assous M, Grimont PA. 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int J Syst Bacteriol 42:378-383. doi: 10.1099/00207713-42-3-378.
86. Margos G, Vollmer SA, Cornet M, Garnier M, Fingerle V, Wilske B, Bormane A, Vitorino L, Collares-Pereira M, Drancourt M. 2009. A new *Borrelia* species defined by multilocus sequence analysis of housekeeping genes. Applied and environmental microbiology 75:5410-5416. doi: 10.1128/AEM.00116-09.
87. Busch U, Hizo-Teufel C, Boehmer R, Fingerle V, Nitschko H, Wilske B, Preac-Mursic V. 1996. Three species of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* sensu stricto, *B afzelii*, and *B. garinii*) identified from cerebrospinal fluid isolates by pulsed-field gel electrophoresis and PCR. Journal of Clinical Microbiology 34:1072-1078. doi: 10.1128/jcm.34.5.1072-1078.1996.
88. Radolf JD, Strle K, Lemieux JE, Strle F. 2021. Lyme Disease in Humans. Curr Issues Mol Biol 42:333-384. doi: 10.21775/cimb.042.333.
89. Balmelli T, Piffaretti JC. 1995. Association between different clinical manifestations of Lyme disease and different species of *Borrelia burgdorferi* sensu lato. Research in Microbiology 146:329-340. doi: 10.1016/0923-2508(96)81056-4.
90. Coipan EC, Jahfari S, Fonville M, Oei GA, Spanjaard L, Takumi K, Hovius JW, Sprong H. 2016. Imbalanced presence of *Borrelia burgdorferi* s.l. multilocus sequence types in clinical manifestations of Lyme borreliosis. Infect Genet Evol 42:66-76. doi: 10.1016/j.meegid.2016.04.019.
91. van Dam AP, Kuiper H, Vos K, Widjojokusumo A, de Jongh BM, Spanjaard L, Ramselaar AC, Kramer MD, Dankert J. 1993. Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. Clinical Infectious Diseases 17:708-717. doi: 10.1093/clinids/17.4.708.
92. Callister SM, Jobe DA, Stuparic-Stancic A, Miyamasu M, Boyle J, Dattwyler RJ, Arnaboldi PM. 2016. Detection of IFN-gamma secretion by T cells collected before and after successful treatment of early Lyme disease. Clin Infect Dis 62:1235-1241. doi: 10.1093/cid/ciw112.

93. Baarsma ME, van de Schoor FR, Van den Wijngaard CC, Joosten LAB, Kullberg BJ, Hovius JW. 2021. The Initial QuantiFERON-Lyme Prototype is Unsuitable for European Patients. Clin Infect Dis 73:1125-1126. doi: 10.1093/cid/ciab254.
94. Nordberg M, Forsberg P, Nyman D, Skogman BH, Nyberg C, Ernerudh J, Eliasson I, Ekerfelt C. 2012. Can ELISpot be applied to a clinical setting as a diagnostic utility for neuroborreliosis? Cells 1:153-67. doi: 10.3390/cells1020153.
95. Widhe M, Ekerfelt C, Jarefors S, Skogman BH, Peterson EM, Bergstrom S, Forsberg P, Ernerudh J. 2009. T-cell epitope mapping of the *Borrelia garinii* outer surface protein A in Lyme neuroborreliosis. Scand J Immunol 70:141-8. doi: 10.1111/j.1365-3083.2009.02285.x.
96. Forsberg P, Ernerudh J, Ekerfelt C, Roberg M, Vrethem M, Bergstrom S. 1995. The outer surface proteins of Lyme disease *Borrelia* spirochetes stimulate T cells to secrete interferon-gamma (IFN-gamma): diagnostic and pathogenic implications. Clin Exp Immunol 101:453-60. doi: 10.1111/j.1365-2249.1995.tb03134.x.
97. von Baehr V, Doebis C, Volk HD, von Baehr R. 2012. The lymphocyte transformation test for *Borrelia* detects active Lyme borreliosis and verifies effective antibiotic treatment. Open Neurol J 6:104-12. doi: 10.2174/1874205X01206010104.
98. Glickstein L, Moore B, Bledsoe T, Damle N, Sikand V, Steere AC. 2003. Inflammatory cytokine production predominates in early Lyme disease in patients with erythema migrans. Infect Immun 71:6051-3. doi: 10.1128/IAI.71.10.6051-6053.2003.
99. Kaiser R, Rauer S. 1998. Analysis of the intrathecal immune response in neuroborreliosis to a sonicate antigen and three recombinant antigens of *Borrelia burgdorferi* sensu stricto. Eur J Clin Microbiol Infect Dis 17:159-66. doi: 10.1007/BF01691111.
100. Skogman BH, Croner S, Forsberg P, Ernerudh J, Lahdenne P, Sillanpaa H, Seppala I. 2008. Improved laboratory diagnostics of Lyme neuroborreliosis in children by detection of antibodies to new antigens in cerebrospinal fluid. Pediatr Infect Dis J 27:605-12. doi: 10.1097/INF.0b013e31816a1e29.
101. Wilske B. 2005. Epidemiology and diagnosis of Lyme borreliosis. Ann Med 37:568-79. doi: 10.1080/07853890500431934.
102. Panelius J, Lahdenne P, Saxen H, Carlsson SA, Heikkila T, Peltomaa M, Lauhio A, Seppala I. 2003. Diagnosis of Lyme neuroborreliosis with antibodies to recombinant proteins DbpA, BBK32, and OspC, and VlsE IR6 peptide. J Neurol 250:1318-27. doi: 10.1007/s00415-003-0205-2.
103. Marangoni A, Moroni A, Accardo S, Cevenini R. 2008. *Borrelia burgdorferi* VlsE antigen for the serological diagnosis of Lyme borreliosis. Eur J Clin Microbiol Infect Dis 27:349-54. doi: 10.1007/s10096-007-0445-7.
104. Liang FT, Yan J, Mbow ML, Sviat SL, Gilmore RD, Mamula M, Fikrig E. 2004. *Borrelia burgdorferi* changes its surface antigenic expression in response to host immune responses. Infect Immun 72:5759-67. doi: 10.1128/IAI.72.10.5759-5767.2004.
105. Rupprecht TA, Koedel U, Fingerle V, Pfister HW. 2008. The pathogenesis of Lyme neuroborreliosis: from infection to inflammation. Mol Med 14:205-12. doi: 10.2119/2007-00091.Rupprecht.
106. Verhaegh D, Joosten LAB, Oosting M. 2017. The role of host immune cells and *Borrelia burgdorferi* antigens in the etiology of Lyme disease. Eur Cytokine Netw 28:70-84. doi: 10.1684/ecn.2017.0396.
107. Moniuszko A, Czupryna P, Pancewicz S, Rutkowski K, Zajkowska O, Swierzbinska R, Grygorczuk S, Kondrusik M, Owlasiuk P, Zajkowska J. 2014. Evaluation of CXCL8, CXCL10, CXCL11, CXCL12 and CXCL13 in serum and cerebrospinal fluid of patients with neuroborreliosis. Immunol Lett 157:45-50. doi: 10.1016/j.imlet.2013.11.002.
108. Cerar T, Ogrinc K, Lotric-Furlan S, Kobal J, Levicnik-Stezinar S, Strle F, Ruzic-Sabljic E. 2013. Diagnostic value of cytokines and chemokines in Lyme neuroborreliosis. Clin Vaccine

Immunol 20:1578-84. doi: 10.1128/CVI.00353-13.
109. Gyllemark P, Forsberg P, Ernerudh J, Henningsson AJ. 2017. Intrathecal Th17-and B cell-associated cytokine and chemokine responses in relation to clinical outcome in Lyme neuroborreliosis: a large retrospective study. Journal of Neuroinflammation 14:1-9. doi: 10.1186/s12974-017-0789-6.
110. Pietikäinen A, Maksimow M, Kauko T, Hurme S, Salmi M, Hytönen J. 2016. Cerebrospinal fluid cytokines in Lyme neuroborreliosis. Journal of neuroinflammation 13:1-10. doi: 10.1186/s12974-016-0745-x.
111. Skogman BH, Lager M, Brudin L, Jenmalm MC, Tjernberg I, Henningsson AJ. 2020. Cytokines and chemokines in cerebrospinal fluid in relation to diagnosis, clinical presentation and recovery in children being evaluated for Lyme neuroborreliosis. Ticks and tick-borne diseases 11:101390. doi: 10.1016/j.ttbdis.2020.101390.
112. Grygorczuk S, Czupryna P, Dunaj J, Moniuszko-Malinowska A, Świerzbińska R, Pancewicz S. 2021. The chemotactic cytokines in the cerebrospinal fluid of patients with neuroborreliosis. Cytokine 142:155490. doi: 10.1016/j.cyto.2021.155490.
113. Millington KA, Innes JA, Hackforth S, Hinks TS, Deeks JJ, Dosanjh DP, Guyot-Revol V, Gunatheesan R, Klenerman P, Lalvani A. 2007. Dynamic relationship between IFN-γ and IL-2 profile of *Mycobacterium tuberculosis*-specific T cells and antigen load. The Journal of Immunology 178:5217-5226. doi: 10.4049/jimmunol.178.8.5217.
114. Krummel B, Strassburg A, Ernst M, Reiling N, Eker B, Rath H, Hoerster R, Wappler W, Glaewe A, Schoellhorn V. 2010. Potential role for IL-2 ELISpot in differentiating recent and remote infection in tuberculosis contact tracing. PLoS One 5:e11670. doi: 10.1371/journal.pone.0011670.
115. Chiappini E, Della Bella C, Bonsignori F, Sollai S, Amedei A, Galli L, Niccolai E, Del Prete G, Singh M, D'Elios MM, de Martino M. 2012. Potential role of *M. tuberculosis* specific IFN-gamma and IL-2 ELISPOT assays in discriminating children with active or latent tuberculosis. PLoS One 7:e46041. doi: 10.1371/journal.pone.0046041.
116. Ekerfelt C, Ernerudh J, Bunikis J, Vrethem M, Aagesen J, Roberg M, Bergstrom S, Forsberg P. 1997. Compartmentalization of antigen specific cytokine responses to the central nervous system in CNS borreliosis: secretion of IFN-gamma predominates over IL-4 secretion in response to outer surface proteins of Lyme disease *Borrelia* spirochetes. J Neuroimmunol 79:155-62. doi: 10.1016/s0165-5728(97)00118-5.
117. Widhe M, Skogman BH, Jarefors S, Eknefelt M, Eneström G, Nordwall M, Ekerfelt C, Croner S, Bergström S, Forsberg P. 2005. Up-regulation of *Borrelia*-specific IL-4-and IFN-γ-secreting cells in cerebrospinal fluid from children with Lyme neuroborreliosis. International Immunology 17:1283-1291. doi: 10.1093/intimm/dxh304.
118. Ogrinc K, Lusa L, Lotrič-Furlan S, Bogovič P, Stupica D, Cerar T, Ružić-Sabljić E, Strle F. 2016. Course and outcome of early European Lyme neuroborreliosis (Bannwarth syndrome): clinical and laboratory findings. Reviews of Infectious Diseases 63:346-353. doi: 10.1093/cid/ciw299.
119. Barabas S, Spindler T, Kiener R, Tonar C, Lugner T, Batzilla J, Bendfeldt H, Rascle A, Asbach B, Wagner R, Deml L. 2017. An optimized IFN-gamma ELISpot assay for the sensitive and standardized monitoring of CMV protein-reactive effector cells of cell-mediated immunity. BMC Immunol 18:14. doi: 10.1186/s12865-017-0195-y.
120. Jafari C, Ernst M, Kalsdorf B, Greinert U, Diel R, Kirsten D, Marienfeld K, Lalvani A, Lange C. 2006. Rapid diagnosis of smear-negative tuberculosis by bronchoalveolar lavage enzyme-linked immunospot. Am J Respir Crit Care Med 174:1048-54. doi: 10.1164/rccm.200604-465OC.
121. Limonard GJ, Thijsen SF, Bossink AW, Asscheman A, Bouwman JJ. 2012. Developing a new clinical tool for diagnosing chronic Q fever: the Coxiella ELISPOT. FEMS Immunol Med Microbiol 64:57-60. doi: 10.1111/j.1574-695X.2011.00890.x.

122. van Gemert JP, Thijsen SFT, Bossink AW. 2011. Tuberculosis-specific T-cell response after recent treatment and remote cure. Eur Respir J 38:1225-8. doi: 10.1183/09031936.00170010.
123. Greissl J, Pesesky M, Dalai SC, Rebman AW, Soloski MJ, Horn EJ, Dines JN, Gittelman RM, Snyder TM, Emerson RO. 2021. Immunosequencing of the T-cell receptor repertoire reveals signatures specific for diagnosis and characterization of early Lyme disease. medRxiv.
124. Luft BJ, Dunn JJ, Dattwyler RJ, Gorgone G, Gorevic PD, Schubach WH. 1993. Cross-reactive antigenic domains of the flagellin protein of *Borrelia burgdorferi*. Res Microbiol 144:251-7. doi: 10.1016/0923-2508(93)90009-q.
125. Kaiser R. 1995. Intrathecal immune response in neuroborreliosis: importance of cross-reactive antibodies. Zentralbl Bakteriol 282:303-14. doi: 10.1016/s0934-8840(11)80131-3.
126. Hoeve-Bakker BJA, Jonker M, Brandenburg AH, den Reijer PM, Stelma FF, van Dam AP, van Gorkom T, Kerkhof K, Thijsen SFT, Kremer K. 2022. The Performance of Nine Commercial Serological Screening Assays for the Diagnosis of Lyme Borreliosis: a Multicenter Modified Two-Gate Design Study. Microbiology Spectrum 10:e00510-22. doi: 10.1128/spectrum.00510-22.
127. Magnarelli LA, Miller JN, Anderson JF, Riviere GR. 1990. Cross-reactivity of nonspecific treponemal antibody in serologic tests for Lyme disease. Journal of clinical microbiology 28:1276-1279. doi: 10.1128/jcm.28.6.1276-1279.1990.
128. Magnarelli LA, Anderson JF, Johnson RC. 1987. Cross-reactivity in serological tests for Lyme disease and other spirochetal infections. J Infect Dis 156:183-8. doi: 10.1093/infdis/156.1.183.
129. Koetsveld J, Platonov AE, Kuleshov K, Wagemakers A, Hoornstra D, Ang W, Szekeres S, van Duijvendijk GLA, Fikrig E, Embers ME, Sprong H, Hovius JW. 2020. *Borrelia miyamotoi* infection leads to cross-reactive antibodies to the C6 peptide in mice and men. Clin Microbiol Infect 26:513 e1-513 e6. doi: 10.1016/j.cmi.2019.07.026.
130. Luther B, Moskophidis M. 1990. Antigenic cross-reactivity between *Borrelia burgdorferi*, *Borrelia recurrentis*, *Treponema pallidum*, and *Treponema phagedenis*. Zentralbl Bakteriol 274:214-26. doi: 10.1016/s0934-8840(11)80104-0.
131. Raffetin A, Saunier A, Bouiller K, Caraux-Paz P, Eldin C, Gallien S, Jouenne R, Belkacem A, Salomon J, Patey O, Talagrand-Reboul E, Jaulhac B, Grillon A. 2020. Unconventional diagnostic tests for Lyme borreliosis: a systematic review. Clin Microbiol Infect 26:51-59. doi: 10.1016/j.cmi.2019.06.033.
132. Ekerfelt C, Forsberg P, Svenvik M, Roberg M, Bergstrom S, Ernerudh J. 1999. Asymptomatic *Borrelia*-seropositive individuals display the same incidence of *Borrelia*-specific interferon-gamma (IFN-gamma)-secreting cells in blood as patients with clinical *Borrelia* infection. Clin Exp Immunol 115:498-502. doi: 10.1046/j.1365-2249.1999.00840.x.
133. Ekerfelt C, Masreliez C, Svenvik M, Ernerudh J, Roberg M, Forsberg P. 2001. Antibodies and T-cell reactivity to *Borrelia burgdorferi* in an asymptomatic population: a study of healthy blood donors in an inland town district in the south-east of Sweden. Scand J Infect Dis 33:806-8. doi: 10.1080/00365540110077376.
134. Dahl V, Wisell KT, Giske CG, Tegnell A, Wallensten A. 2019. Lyme neuroborreliosis epidemiology in Sweden 2010 to 2014: clinical microbiology laboratories are a better data source than the hospital discharge diagnosis register. Euro Surveill 24. doi: 10.2807/1560-7917.ES.2019.24.20.1800453.
135. Nordberg CL, Bodilsen J, Knudtzen FC, Storgaard M, Brandt C, Wiese L, Hansen BR, Andersen AB, Nielsen H, Lebech AM, group Ds. 2020. Lyme neuroborreliosis in adults: A nationwide prospective cohort study. Ticks Tick Borne Dis 11:101411. doi: 10.1016/j.ttbdis.2020.101411.

136. Knudtzen FC, Andersen NS, Jensen TG, Skarphedinsson S. 2017. Characteristics and Clinical Outcome of Lyme Neuroborreliosis in a High Endemic Area, 1995-2014: A Retrospective Cohort Study in Denmark. Clin Infect Dis 65:1489-1495. doi: 10.1093/cid/cix568.
137. Schwenkenbecher P, Pul R, Wurster U, Conzen J, Pars K, Hartmann H, Suhs KW, Sedlacek L, Stangel M, Trebst C, Skripuletz T. 2017. Common and uncommon neurological manifestations of neuroborreliosis leading to hospitalization. BMC Infect Dis 17:90. doi: 10.1186/s12879-016-2112-z.
138. Strle F, Wormser GP, Mead P, Dhaduvai K, Longo MV, Adenikinju O, Soman S, Tefera Y, Maraspin V, Lotrič-Furlan S. 2013. Gender disparity between cutaneous and non-cutaneous manifestations of Lyme borreliosis. PloS one 8:e64110. doi: 10.1371/journal.pone.0064110.
139. Henningsson AJ, Malmvall BE, Ernerudh J, Matussek A, Forsberg P. 2010. Neuroborreliosis-an epidemiological, clinical and healthcare cost study from an endemic area in the south-east of Sweden. Clin Microbiol Infect 16:1245-51. doi: 10.1111/j.1469-0691.2009.03059.x.
140. Stanek G, Wormser GP, Gray J, Strle F. 2012. Lyme borreliosis. Lancet 379:461-73. doi: 10.1016/S0140-6736(11)60103-7.
141. Ware Jr JE, Sherbourne CD. 1992. The MOS 36-item short-form health survey (SF-36): I. Conceptual framework and item selection. Medical care:473-483.
142. van de Schoor FR, Baarsma ME, Gauw SA, Joosten LAB, Kullberg BJ, van den Wijngaard CC, Hovius JW. 2019. Validation of cellular tests for Lyme borreliosis (VICTORY) study. BMC Infect Dis 19:732. doi: 10.1186/s12879-019-4323-6.
143. Stanek G, Strle F. 2018. Lyme borreliosis-from tick bite to diagnosis and treatment. FEMS Microbiol Rev 42:233-258. doi: 10.1093/femsre/fux047.
144. Santin M, Munoz L, Rigau D. 2012. Interferon-gamma release assays for the diagnosis of tuberculosis and tuberculosis infection in HIV-infected adults: a systematic review and meta-analysis. PLoS One 7:e32482. doi: 10.1371/journal.pone.0032482.
145. Simsek H, Alpar S, Ucar N, Aksu F, Ceyhan I, Gozalan A, Cesur S, Ertek M. 2010. Comparison of tuberculin skin testing and T-SPOT.TB for diagnosis of latent and active tuberculosis. Jpn J Infect Dis 63:99-102. doi: 10.7883/yoken.63.99.
146. Kim JY, Kang YA, Park JH, Cha HH, Jeon NY, Lee SW, Lee SO, Choi SH, Kim YS, Woo JH, Kim SH. 2020. An IFN-gamma and TNF-alpha dual release fluorospot assay for diagnosing active tuberculosis. Clin Microbiol Infect 26:928-934. doi: 10.1016/j.cmi.2019.11.003.
147. Delemarre EM, van Hoorn L, Bossink AWJ, Drylewicz J, Joosten SA, Ottenhoff THM, Akkerman OW, Goletti D, Petruccioli E, Navarra A, van den Broek BTA, Paardekooper SPA, van Haeften I, Koenderman L, Lammers JJ, Thijsen SFT, Hofland RW, Nierkens S. 2021. Serum Biomarker Profile Including CCL1, CXCL10, VEGF, and Adenosine Deaminase Activity Distinguishes Active From Remotely Acquired Latent Tuberculosis. Front Immunol 12:725447. doi: 10.3389/fimmu.2021.725447.
148. Leth TA, Dessau RB, Moller JK. 2022. Discriminating between Lyme neuroborreliosis and other central nervous system infections by use of biomarkers CXCL13 and IL-6. Ticks Tick Borne Dis 13:101984. doi: 10.1016/j.ttbdis.2022.101984.
149. Fitzgerald BL, Graham B, Delorey MJ, Pegalajar-Jurado A, Islam MN, Wormser GP, Aucott JN, Rebman AW, Soloski MJ, Belisle JT, Molins CR. 2021. Metabolic Response in Patients With Post-treatment Lyme Disease Symptoms/Syndrome. Clin Infect Dis 73:e2342-e2349. doi: 10.1093/cid/ciaa1455.
150. Yao Y, Zia A, Neumann RS, Pavlovic M, Balaban G, Lundin KE, Sandve GK, Qiao S-W. 2021. T cell receptor repertoire as a potential diagnostic marker for celiac disease. Clinical Immunology 222:108621. doi: 10.1016/j.clim.2020.108621.
151. Li N, Yuan J, Tian W, Meng L, Liu Y. 2020. T-cell receptor repertoire analysis for the

diagnosis and treatment of solid tumor: A methodology and clinical applications. Cancer Commun (Lond) 40:473-483. doi: 10.1002/cac2.12074.
152. Gittelman RM, Lavezzo E, Snyder TM, Zahid HJ, Elyanow R, Dalai S, Kirsch I, Baldo L, Manuto L, Franchin E. 2021. Diagnosis and tracking of SARS-CoV-2 infection by T-Cell receptor sequencing. medRxiv: 202011 0920228023. doi: 10.1101/2020.11.09.20228023.

8