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Immunodiagnostics of Lyme neuroborreliosis

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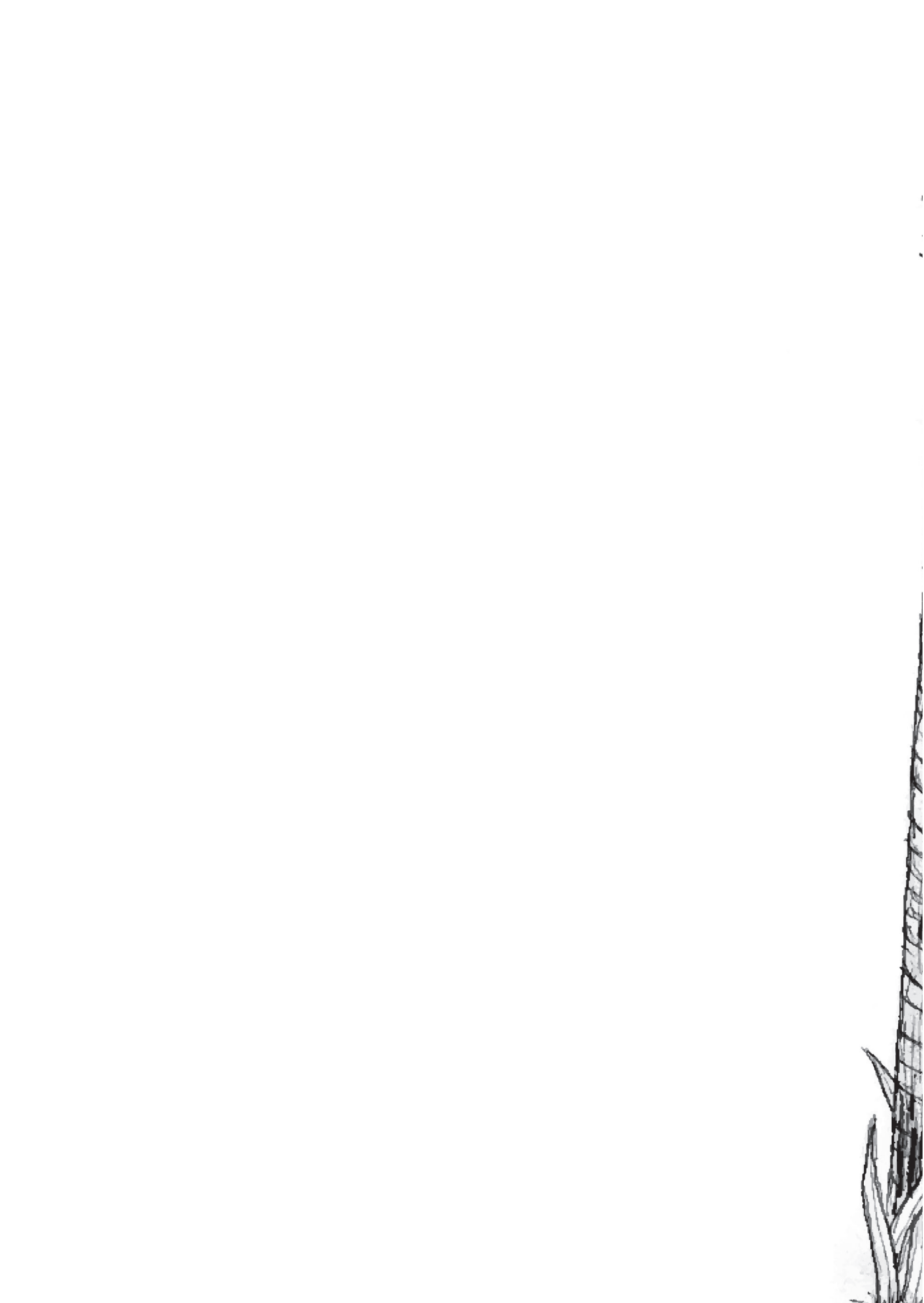

9 SUMMARY

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

CONCLUSION

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

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

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

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