Diagnostic Microbiology and Infectious Disease 104 (2022) 115782

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Original Article

Borrelia burgdorferi specific serum and cerebrospinal fluid antibodies in Lyme neuroborreliosis

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ARTICLE INFO

Article history:
Received 21 March 2022
Revised in revised form 6 June 2022
Accepted 18 July 2022
Available online 22 July 2022

Keywords:
Borrelia burgdorferi
Lyme borreliosis
Lyme neuroborreliosis
Serology
Cerebrospinal fluid

ABSTRACT

We used definite Lyme neuroborreliosis (LNB) adult patient acute and convalescent phase serum (n = 63 and 61, respectively) and cerebrospinal fluid (CSF; acute n = 63, 3 weeks timepoint n = 41) samples to characterize *Borrelia burgdorferi* specific antibody responses in patient subgroups categorized by demographics, infection manifestation and phase, infecting *B. burgdorferi* genospecies, received antibiotic treatments, and treatment outcome. *B. burgdorferi* antibodies were analyzed using 4 different assays incorporating a large array of antigens. We observed that *B. burgdorferi* specific serum antibodies show a universal, antigen independent declining trend after antibiotic treatment of LNB at 1 year. Antibodies declined similarly among women and men over time, and the decline was independent of patient age. The antibody responses were independent of the predominant LNB manifestation, treatment received by the patient, infecting *B. burgdorferi* genospecies, or the subjective improvement experienced by the patients. Finally, the antibody specificities in CSF reflected the specificities observed in serum samples.

1. Introduction

Lyme borreliosis (LB) is a multisystemic zoonosis caused by *Borrelia burgdorferi* sensu lato spirochetes (later *Borrelia*) transmitted through the bites of infected *Ixodes* ticks [1,2]. Early LB manifests as a skin rash called erythema migrans (EM) at tick bite site, while in the later stages of the infection, *Borrelia* may disseminate to various organs. The disseminated form of LB affecting the nervous system is known as Lyme neuroborreliosis (LNB). The most common *Borrelia* genospecies causing LB in North America is *B. burgdorferi* sensu stricto, while in Europe and Asia, *B. garinii* and *B. afzelii* dominate. Occasionally, also some other genospecies, for example, *B. bavariensis and B. spielmanii* have been identified in LB patients. Interestingly, different *Borrelia* genospecies have been linked to manifestations in different organ systems with *B. garinii* being especially associated with LNB in Europe, although other genospecies may also cause LNB [3].

The diagnosis of EM is clinical, while the diagnosis of LNB, along with the other forms of disseminated LB, relies on laboratory testing including serology [4,5]. However, serological methods are poor in discriminating an active infection from a previous episode of LB due to the persistence of *Borrelia* specific antibodies also after adequate antibiotic treatment [6]. There are also obvious gaps in our understanding of the factors affecting the nature and kinetics of the antibody response of LB patients, which may be influenced, for example, by the age and sex of the patient, the infecting *Borrelia* genospecies, and received treatments. Further unresolved issues are whether the antibody response varies in different manifestations of LNB (facial nerve palsy, radiculitis etc.), or whether the clinical improvement experienced by the patient is reflected in the kinetics of the response. In the present study, serum and cerebrospinal fluid samples (CSF) of our well-characterized LNB patient material were analyzed using a large array of *Borrelia* serology methods to fulfill these knowledge gaps.

2. Materials and methods

2.1. Characteristics of the LNB patient samples

Patient samples for the present study were originally collected in our previously published LNB treatment study comparing intravenous ceftriaxone and oral doxycycline [7]. In this study, informed consent was obtained from every patient, and ethical approval was provided by the National Committee on Medical Research Ethics in Finland. The subjects were classified as definite/possible LNB patients

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https://doi.org/10.1016/j.diagmicrobio.2022.115782

Table 1
Baseline demographics and clinical characteristics of the patients included in the study.

		Female (n = 20)	Male (n = 43)	Total (n = 63)
	Age[a]	62.5 (18–88)	58 (28–79)	61 (18–88)
	CSF leukocytes /μL[a]	123.5 (3–530)	79.0 (7–985)	104.0 (3–985)
	IT index IgM[a,b]	1.5 (<0.3–55.6)	1.6 (<0.3–33.9)	1.6 (<0.3–55.6)
	IT index IgG[a,b]	8.1 (<0.3–158.4)	2.6 (<0.3–150.2)	4.4 (<0.3–158.4)
Manifestation	Radiculitis	11 (55%)	10 (23.3%)	21 (33.3%)
	Facial nerve palsy	4 (20%)	11(25.6%)	15 (23.8%)
	Both manifestations	5 (25%)	14 (32.6%)	19 (30.2%)
	Neither manifestation[c]	0 (0%)	8 (18.6%)	8 (12.7%)
Treatment	Intravenous ceftriaxone	7 (35%)	23[d] (53.5%)	30 (47.6%)
	Oral doxycycline	13[e] (65%)	20[e] (46.5%)	33 (52.4%)

[a] Median (range).
[b] IDEIA Lyme Neuroborreliosis test (reference range <0.3).
[c] For example, headache, neck-shoulder pain, neck stiffness, other cranial nerve palsy, myalgia, arthralgia, fatigue, vertigo.
[d] Treatment modification in 2 male patients.
[e] Treatment modification in 1 male and 1 female patient.

using the criteria by EFNS [8], where IDEIA LNB test (Oxoid, Basingstoke, UK) was used to demonstrate intrathecal borrelia-antibody production.

Sixty-three definite LNB patients with a full or nearly full set of samples were included in the present study (Supplementary Table S1). There were 20 female and 43 male patients and their median age was 61 years (Table 1). The major manifestations of the patients and the treatments received by the patients are indicated in Table 1. According to the study protocol of the original study, the antibiotic treatment of 4 patients was modified, and therefore, in the present study these patients/samples were excluded from the analyses concerning the kinetics of antibodies following the treatments.

Serum samples were collected at the time of diagnosis, and 3 weeks, 4 months, and 12 months after the diagnosis. CSF samples were collected at the time of diagnosis before the initiation of antibiotic treatment and, in a subgroup of patients (n = 41; collected as per the study protocol of the original study if a patient had ≥50 leukocytes/μL in the first CSF sample) CSF samples were available also from 3 weeks' time point (Supplementary Table S1). After collection, samples were stored at -20 °C, and freeze-thaw cycles were limited to minimum (3–4 cycles).

2.2. Borrelia *serology assays*

Antibodies to *Borrelia* in the serum were analyzed using 4 different assays, while CSF samples were analyzed with 3 methods (Supplementary Table S1).

In-house ELISA IgM and IgG assays were performed as previously described [9]. Briefly, microtiter plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with whole cell sonicate (WCS) antigen prepared from *B. burgdorferi* sensu stricto B31 at 37 °C overnight. The wells were blocked with PBS containing 1% normal sheep serum (1% NSS-PBS) at 37 °C for 1 hour, after which diluted samples were added to the wells and incubated at 37 °C for 2 hours. After washings, goat anti-human alkaline phosphate conjugated secondary antibodies (Calbiochem, San Diego, CA, USA) were incubated in the wells at 37 °C for 2 hours, substrate solution (diethanolamine, Reagena, Finland) was added, the reaction was stopped with NaOH, and the absorbance values were measured and the results calculated as enzyme-immunoassay units (EIU) with the BEP III System (Siemens Healthcare GmbH, Germany).

LIAISON®BorreliaIgM Quant and IgG assays (Diasorin, Italy) are indirect chemiluminescence immunoassays (CLIA) with recombinant antigens (OspC and VlsE for detection of IgM and VlsE for IgG). The assays were performed using Liaison® XL equipment according to the instructions by the manufacturer, and the results are expressed as AU/mL units. CSF samples were not analyzed with this assay.

C6 Lyme ELISA™ (Immunetics/Oxford Immunotech, Boston, MA), where the antigen is the C6-peptide derived of the VlsE *Borrelia* surface protein, was performed as instructed by the manufacturer, and absorbances were measured with Multiskan GO (Thermo Scientific, Vantaa, Finland). CSF samples were analyzed using the protocol by the manufacturer with the exception that the samples were diluted 1:5 as previously described [10]. The results are expressed as Lyme index units.

*recom*BeadBorreliaIgM and IgG (Mikrogen, Germany) multi-antigen bead-immunoassay analyses were performed as instructed by the manufacturer unless otherwise indicated. The assay includes following antigens coated separately on the beads: p100, VlsE, p58, p39, OspA, OspC *B. burgdorferi* ss (OspC Bbss), OspC *B. afzelii* (OspC Ba), OspC *B. garinii* (OspC Bg), p18 *B. burgdorferi* ss (p18 Bbss), p18 *B. afzelii* (p18 Ba), p18 *B. bavariensis* (p18 Bbav), p18 *B. garinii* (p18 Bg), p18 *B. spielmanii* (p18 Bsp). Antigen specific antibody binding signal intensities (cut-off-index; COI) and the overall result score provided by the analysis software were considered separately in the statistical analysis.

When determining the infecting *Borrelia* genospecies with the help of serum and CSF sample p18 (DbpA) IgM and IgG antibody reactivity, the following criteria developed for this study were used as indication of the genospecies: COI was 0.3 or higher against p18 of a genospecies, and the COI was at least 3 times higher than the COI-values of any other p18 reactivity.

In addition, COI 0.3 was considered as a cut-off for a positive reaction when the reactivities against *recom*Bead antigens detected in the CSF sample of individual patient were compared with those observed in the serum sample of the same subject.

2.3. Statistics and data management

Data collection and pre-processing was performed by Microsoft Excel 2016 (Redmond, WA). Data was anonymized after the collection according to the GDPR regulations. The data was analyzed using IBM SPSS Statistics for Windows, Version 27.0 (Armonk, NY). The distribution of the data was analyzed with Shapiro-Wilk test. If the data was normally distributed, comparisons between timepoints 0 and 12 months were performed with paired samples t-test, and comparisons between different groups at a specific timepoint were performed with independent samples t-test (if there were only 2 variables), or with 1-way ANOVA adjusted by the Tukey Post Hoc correction (multiple comparisons). If the data was not normally distributed, comparisons between timepoints 0 and 12 months were performed with related-samples Wilcoxon signed rank test and comparisons between different groups at a specific timepoint were performed with Mann-Whitney U test (if there were only 2 variables), or with Kruskal-

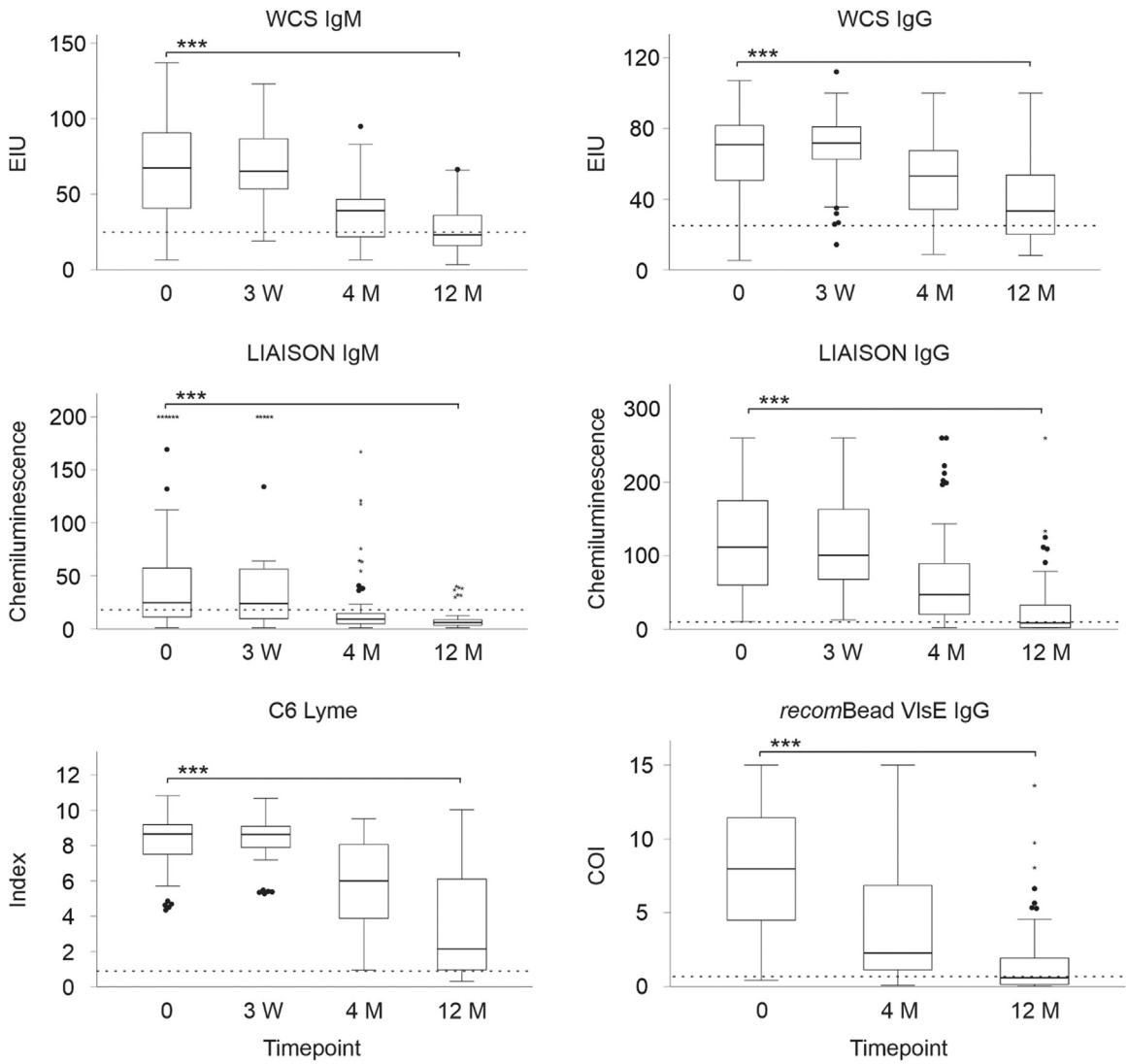

Fig. 1. Serum IgG and IgM antibody kinetics in LNB patients. Only patients with samples from all timepoints are included. Box plots show the medians, and 25th and 75th percentiles. Whiskers show the maximum and minimum values excluding outliers. Potential outliers are presented as black dots and extreme outliers as small asterisks. The indicated timepoints are acute phase before treatment initiation (0), 3 weeks (3 W), 4 months (4 M), and 12 months (12 M) after the treatment. *recom*Bead IgG VlsE results are highlighted here, since this specificity was as a rule present in most of the samples, while other serum *recom*Bead IgM/IgG results are shown in Supplementary Fig.S2A and 2B. The dashed lines indicate the cut-off values of each test (WSC 25 EIU; Liaison IgM 18; Liaison IgG 10; C6 Lyme 0.9 index; recomBead VlsE IgG 0.67 COI). *** = $P < 0.001$.

Wallis test adjusted by the Bonferroni correction (multiple comparisons). The differences were regarded as statistically significant if *P*-value was < 0.05. Correlations were analyzed with the Spearman's method (not normally distributed data).

3. Results

3.1. Overview of the kinetics of Borrelia antibodies in sera of LNB patients

In this study, we used 4 different serological methods to analyze *Borrelia* antibody kinetics in serum samples of 63 LNB patients collected at the time of diagnosis, and at 3 weeks, 4 months, and 12 months after the diagnosis (Supplementary Table S1).

The analysis of the overall kinetics of serum antibodies indicated a declining trend in all assays, and the trend was observed in both IgM and IgG antibodies (Fig. 1). The decline was statistically significant with all assays, when timepoints 0 and 12 months were compared. Supplementary Figure S1 shows the longitudinal antibody kinetics of individual patients from 0 to 12 months. The antibodies declined similarly over time among men and women, and the decline was independent of patient age (Supplementary Table S2).

In *recom*Bead IgM assay, significant reactivity was detectable against only a few antigens with OspC dominating (Supplementary Fig. S2A). In *recom*Bead IgG, VlsE was the dominant antigen (Supplementary Fig. S2B), and therefore, VlsE IgG results are highlighted also in Fig. 1 as well as in other main figures. Statistics of the decline of the antibody responses observed in the *recom*Bead assays between 0 and 12 months' time points were calculated concerning antigens with significant IgM and/or IgG reactivity at 0 months (p100/IgG, VlsE/IgG, p58/IgG, OspC/IgM/IgG all genospecies, p18/IgG Bg, and the overall IgM/IgG score). The decline was statistically significant in all occasions.

C6 peptide is derived from the VlsE surface molecule of *Borrelia*. Therefore, we analyzed the correlation of C6 Lyme index with *recom*Bead VlsE IgG COI results (Supplementary Figure S3). There was a strong correlation between C6 Lyme index and VlsE IgG ($r_s = 0.751$, $P < 0.001$).

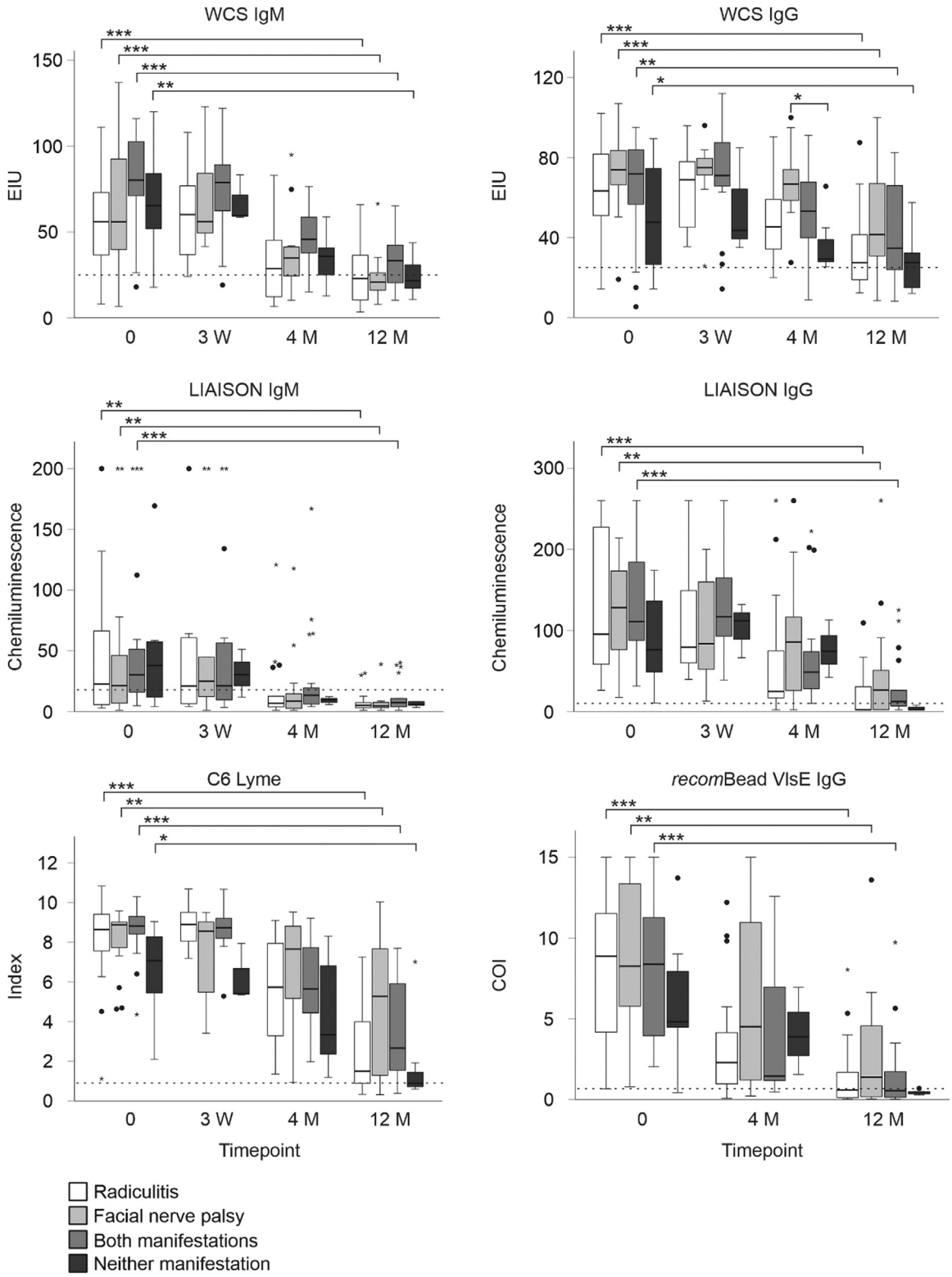

Fig. 2. Serum IgG and IgM antibody kinetics in LNB patients as in Fig. 1, but categorized based on the major presenting symptom of the patients (radiculitis, facial nerve palsy, both of the manifestations, neither of the manifestations). The number of patients in each group is indicated in Table 1. Outliers are presented as black dots and extreme outliers as small asterisks. Statistically significant differences between the timepoints and between the groups at each time point are presented. The dashed lines indicate the cut-off values of each test. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

3.2. Kinetics of antibodies in sera of LNB patients categorized based on the dominant LNB manifestation

The presenting manifestation among the 63 LNB patients was facial nerve palsy in 15 cases and painful radiculitis in 21 cases (Table 1). Nineteen patients had both manifestations, and in 8 patients neither of these manifestations were present. The results in Fig. 2 show that the antibodies declined statistically significantly in all manifestation groups with the exception of the group "Neither manifestation", where no statistical

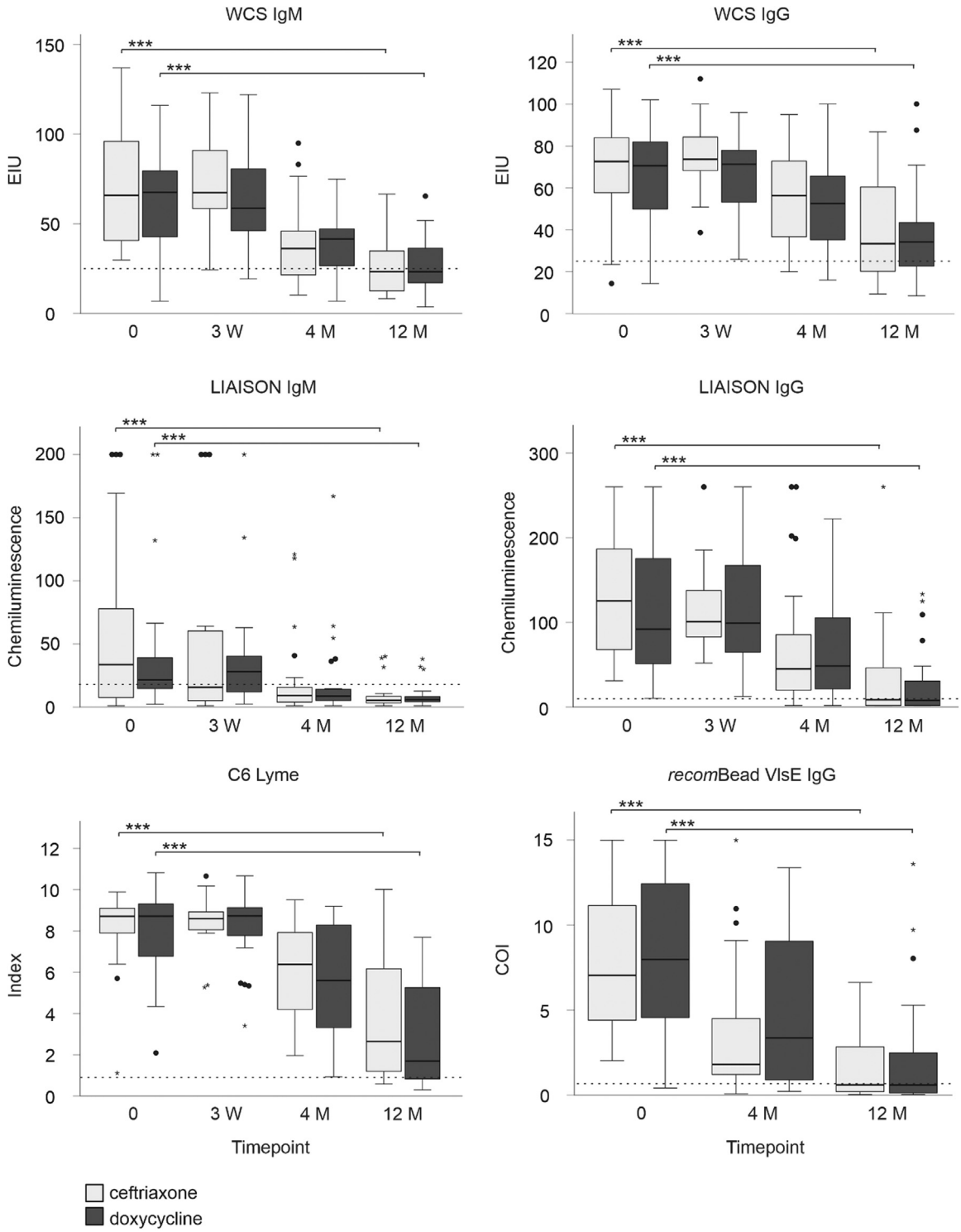

Fig. 3. Serum IgG and IgM antibody kinetics in LNB patients as in Fig. 1, but categorized based on the treatment received by the patients. The number of patients in each group is indicated in Table 1. Outliers are presented as black dots and extreme outliers as small asterisks. Statistically significant differences between the timepoints in each group are presented. The dashed lines indicate the cut-off values of each test. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

significance was reached in Liaison IgM and IgG, and in *recom*-Bead VlsE IgG. Further, when the antibody levels of the different manifestation groups were compared at each time point with the different tests, only the "Facial nerve palsy" and "Neither manifestation" groups differed significantly at 4 months in the WCS IgG assay.

3.3. Kinetics of antibodies in sera of LNB patients treated either with oral doxycycline or intravenous ceftriaxone

In the original study [7], the patients were treated either with oral doxycycline or intravenous ceftriaxone (Table 1). In the present study, we evaluated the kinetics of the *Borrelia* antibody response in

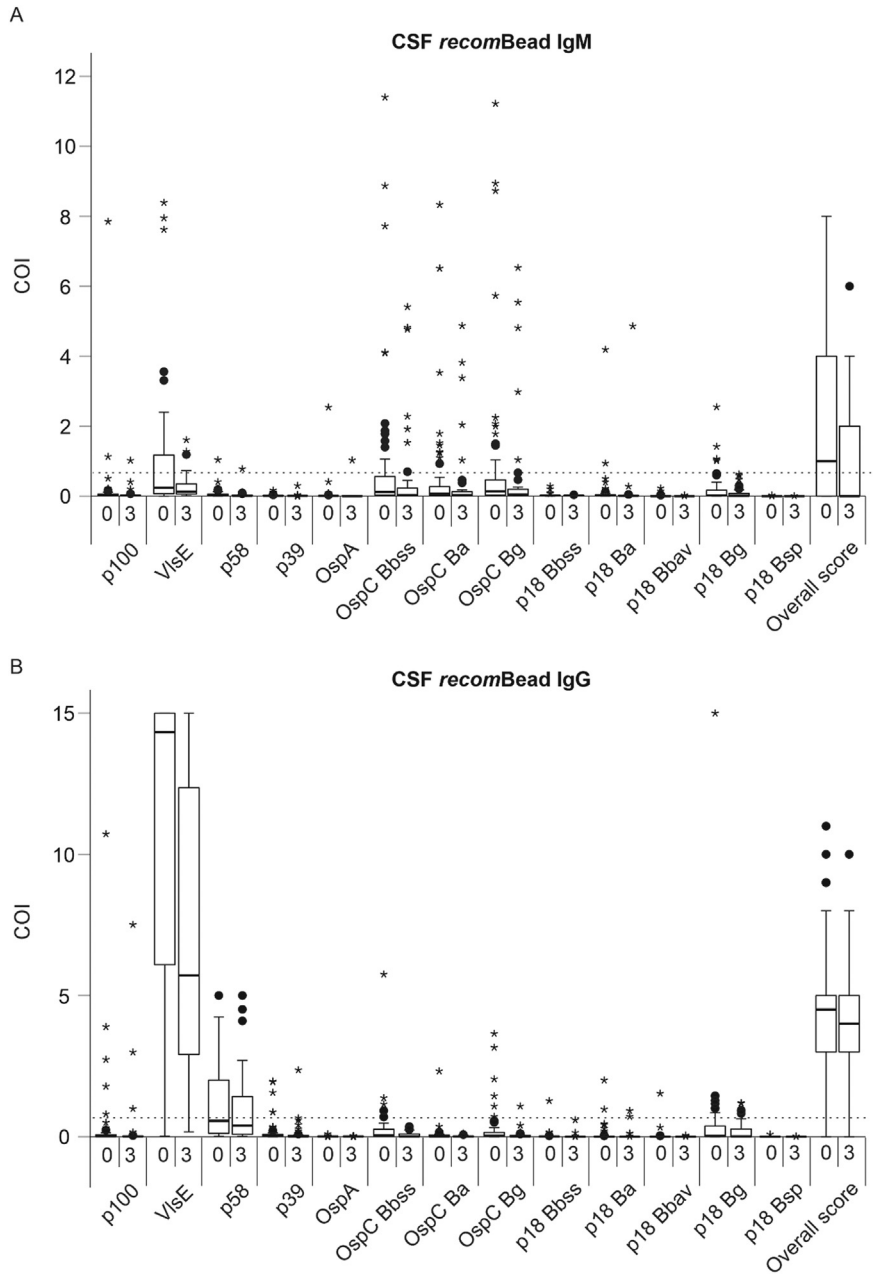

Fig. 4. (A) *recom*Bead IgM and (B) IgG antibodies in CSF and the overall scores at the time of diagnosis and 3 weeks after the diagnosis are shown. The vertical lines inside the box-plots represent median values. Outliers are presented as black dots and extreme outliers as small asterisks. The dashed lines indicate the cut-off values of the tests.

both groups separately (Fig. 3). The results show that the declining antibody trend is identical and statistically significant in both groups with all assays when timepoints 0 and 12 months are compared. Moreover, there are no statistically significant differences between the treatment groups at any time point.

3.4. *Specificity of* Borrelia *antibodies in CSF samples before antibiotic treatment and at 3 weeks after treatment initiation*

Borrelia antibodies in CSF samples collected before the initiation of antibiotic treatment and, in a subgroup of 41 patients, at 3 weeks' time point were analyzed with WCS IgM/IgG assays, C6 Lyme ELISA and *recom*Bead IgM/IgG assays.

The results in Supplementary Fig. S4 show strong antibody IgG reactivity against the WCS and the index value in C6 Lyme is highly elevated. WCS IgM reactivity is moderate. In *recom*Bead IgM assay (Fig. 4A), significant antibody reactivity was detectable mainly against VlsE and OspC antigens, while in *recom*Bead IgG assay, VlsE was the dominant antigen (Fig. 4B). There appears to be no major change in the antibody reactivities between the timepoints 0 and 3 weeks.

When the reactivities against *recom*Bead antigens detected in the CSF sample of an individual patient were compared with those observed in the serum sample of the same subject, it is evident that the reactivities are highly overlapping (Supplementary Table S3). The table is read in such a way that, for example, VlsE reactivity was detected in both CSF and serum samples of 50 individuals, while there were no patients with this antibody specificity only in serum or CSF. p58 reactivity, in turn, was detected both in the CSF and serum samples of 31 individuals, while there were 4 patients with p58 reactivity only in serum and 2 patients with p58 reactivity only in CSF.

As in serum samples, there was a clear correlation between C6 Lyme index and *recom*Bead VlsE IgG COI results (r_s = 0.655, $P < 0.001$).

We also analyzed the CSF antibody reactivities at 0 months against *recom*Bead antigens categorized by the major LNB manifestations (as in Fig. 2); however, no major differences in the antibody specificities among the manifestation groups were observed (data not shown).

3.5. *Identification of the infecting* Borrelia *genospecies*

*recom*Bead IgM and IgG assays include genospecies specific OspC and p18 antigens. We took advantage of the p18 antigen reactivities in sera and CSF samples to identify the probable genospecies of the infecting *Borrelia* bacteria in each patient. According to our scheme, genospecies identification could be made, when there was IgM and/or IgG reactivity (≥ 0.3) against a single p18, which was at least 3 times over the reactivity observed against p18 of any other genospecies (Supplementary Fig. S5). As a result, the infecting genospecies could be presumptively identified in 33/63 patients. The bacteria were identified as *B. garinii* in 22 cases, as *B. afzelii* in 9 cases, as *B. burgdorferi* ss in 1 case, and *B. bavariensis* also in 1 case, while in 30 cases the identification was not possible due to multiple or no reactivity against p18. For statistical analysis, the 3 latter groups were combined.

3.6. *Kinetics of* Borrelia *antibodies categorized by infecting genospecies*

The analysis of the kinetics of serum *Borrelia* antibodies categorized according to the infecting genospecies indicated a declining trend in all assays with all genospecies, and the trend was observed in both IgM and IgG antibodies (Supplementary Fig. S6). However, the decline in antibodies measured with LIAISON IgM and IgG assays did not reach statistical significance with patients which were categorized into *B. afzelii* group. Further, when the antibody levels of the genospecies groups were compared at each time point, statistically significant differences were observed in 2 occasions: in the WCS IgG at 4 months between "*B. garinii*" and "No identification/*B. bavariensis*/*B. burgdorferi*," and in Liaison IgG at 12 months between "*B. afzelii*" and "No identification/B. *bavariensis*/*B. burgdorferi*."

3.7. *The main symptoms of the LNB patients categorized by* Borrelia *genospecies*

As explained above, the main LNB manifestations of the patients were facial nerve palsy and painful radiculitis [7]. Therefore, we next analyzed the distribution of the main manifestations categorized by *Borrelia* genospecies. Based on our data, there are no differences in the distribution of manifestations between *B. afzelii* and *B. garinii* (Supplementary Table S4), and both genospecies associate with radiculitis and facial nerve palsy. Interestingly, LNB patients with neither of the major manifestations were all classified in the no genospecies identification group. Erythema migrans was detected in 20 of 63 patients, and the distribution of this manifestation among the genospecies groups is also indicated in Supplementary Table S4.

3.8. *The subjective outcome of the LNB patients versus* Borrelia *genospecies and antibody levels*

In our original LNB treatment study [7], the patients defined their subjective condition by a visual analogue scale (VAS) on a scale from 0 to 10 (0 = normal, 10 = worst possible) at every visit (before treatment, and at 3 weeks, 4 months, and 12 months). Therefore, we asked the questions whether post-treatment subjective outcome of LNB patients is influenced by the infecting *Borrelia* genospecies, and whether the decline in the VAS score parallels the decline observed in the antibody levels.

The results in Supplementary Fig. S7 indicate no difference in the decline of the VAS score between the genospecies groups. Finally, there was no correlation between the VAS score and the *Borrelia* antibody level at any timepoint (data not shown).

4. Discussion

Diagnostics of LB is hampered by the lack of sensitive direct diagnostic methods (culture or PCR) necessitating the reliance on indirect serologic testing. *Borrelia* serology, in turn, is complicated by insensitivity during the early days of infection due to the physiological window, where antibody response of the host is only starting, while the other major challenge in *Borrelia* serology is the difficulty of differentiating an active infection from a previous episode of LB [4,5]. Although much is known about the immunology of LNB [11] there are still gaps in our understanding of the antigen specific serum and CSF antibody responses in patients subgrouped by, for example, treatments, disease manifestations and outcome, or by the infecting *Borrelia* genospecies.

In the present work, we used acute and convalescent phase serum and CSF samples of our well-characterized LNB treatment study patients [7], along with an array of *Borrelia* serology methods incorporating a large panel of *Borrelia* antigens to identify subgroup specific changes in *Borrelia* antibodies. Noteworthily, our aim was not to evaluate the sensitivity or specificity of the different assays in the diagnostics of LNB. The analysis of the kinetics of *Borrelia* antibodies did not reveal, in any patient group, antigen specificities, which would be uniformly present in the samples collected before treatment, and which would disappear by twelve months after treatment, although in all assays a decreasing antibody trend was noted. Further, there were no differences in the kinetics of antibodies between male and female patients, nor among patients of different age groups. The declining antibody trend was similar in LNB patients with various disease manifestations, and in patients receiving either oral doxycycline or intravenous ceftriaxone treatments. Importantly, antibody levels did not correlate with the subjective well-being of patients (VAS score), further underlining the futility of post-treatment antibody testing in LB.

The most prominent antigen reacting with serum IgM antibodies was OspC, while VlsE overwhelmingly dominated as the target of IgG antibodies. Interestingly, the antibody specificities detected in the CSF samples paralleled those of the sera, and there were only individual CSF samples with *Borrelia* antigen specific antibodies not detected in the serum of the same patient.

C6 peptide is an established and well-performing antigen in LB serology. The molecule is a 25-mer oligopeptide derived from the sixth invariable region (IR_6) of the VlsE surface molecule of *Borrelia*. Based on the crystal structure of VlsE, IR_6 is buried within the tertiary structure of the protein [12], meaning that antibodies reacting with C6 peptide should have minimal access to IR_6 in the intact VlsE. VlsE antibodies likely recognize other antigenic structures and a larger repertoire of epitopes than antibodies directed solely against IR_6/C6 peptide, and thus, it can be speculated that the kinetics of these antibodies is different, for example, post-treatment. However, our results indicate that no such difference exists, and that there is a strong correlation between C6 peptide and VlsE antibodies.

We utilized p18 antigen IgM and IgG reactivities in serum and CSF samples of the LNB patients to identify the presumptive *Borrelia* genospecies of the patient, which led to the identification in 33 of 63 cases with *B. garinii* and *B. afzelii* as the most prevalent genospecies. This finding is in line with the information on the Borrelia genospecies prevalence in ticks in Finland [13], and also with the known tendency of *B. garinii* to cause LNB [14]. However, there was no association of a genospecies with particular manifestations, nor were

there any statistically significant differences in the antibody decline or in the subjective recovery of the LNB patients between the genospecies groups. This is in contrast with a previous study, where patients with *B. garinii* LNB had a distinct clinical presentation compared with LNB caused by *B. afzelii* [15], which may be explained, for example, by the geographic and temporal difference of the 2 studies, or by the different methods used in genospecies identification (serology vs DNA fragment pattern identification with pulsed-field gel electrophoresis).

In conclusion, one major issue in the diagnostics of LNB, and disseminated LB in general, is the difficulty of differentiating active infection from a previous one. The results of the present study further strengthen the view that there are no serologic markers, which would unambiguously identify patients with ongoing LNB and, thus, in need of antibiotic treatment, although the absence of serum IgG VlsE reactivity to some degree argues against acute LNB. Therefore, novel approaches both in the direct detection of *Borrelia*, such as detection of cell-free DNA in human plasma [16], and in non-antibody based indirect methods, such as metabolite-profiling [17], or analysis of leucocyte response [18], are needed.

Ethical approval

This study was performed in line with the principles of the Declaration of Helsinki. Informed consent was obtained from every patient, and ethical approval was provided by the National Committee on Medical Research Ethics in Finland.

Acknowledgments

Tuula Rantasalo is acknowledged for the fundamental support in performing the Liaison and *recom*Bead analyses. Serological lab staff in the Clinical microbiology laboratory of Turku University Hospital is acknowledged for the help and support given for the analyses of this study.

Funding

The study was funded by the Special Governmental Fund for University Hospitals, Turku University Hospital, Sakari Alhopuro Foundation, Orion Research Foundation, the Finnish Medical Foundation and TYKS-Säätiö-Foundation.

Declaration of competing interests

JH is a part time consultant for the diagnostic company Reagena (Toivala, Finland).

Other authors: none.

Author contributions

AP, OG and JH planned the study, and AP and OG performed the statistical analyses. AP, OG and JH wrote the first draft of the manuscript. EK, MK, JO and JH organized and supervised the original LNB treatment study, and the serum and CSF sample collections within that study. All authors have participated in the writing of the manuscript, and read and approved the final version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.diagmicrobio.2022.115782.

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