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Discriminating between Lyme neuroborreliosis and other central nervous system infections by use of biomarkers CXCL13 and IL-6

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ABSTRACT

CXCL13 in cerebrospinal fluid has gradually become an established biomarker for Lyme neuroborreliosis (LNB), however the diagnostic performance of CXCL13 may be improved by the addition of IL-6, a non-specific infection biomarker. The aim of this study was to measure the concentrations of CXCL13 and IL-6 in cerebrospinal fluid, in the attempt to evaluate the diagnostic performance of these two biomarkers, in the differentiation between definite and possible LNB, as well as between LNB and other neuroinfections. This study used a cross-sectional design to quantify the levels of CXCL13 and IL-6 in cerebrospinal fluid (CSF) specimens from consecutive patients examined for central nervous system (CNS) infections at Lillebaelt Hospital in the Region of Southern Denmark. CXCL13 and IL-6 were measured simultaneously using the Bio-Plex 200 multiplex Cytokine Immunoassay System (Bio-Rad). Based on clinical and paraclinical findings, we grouped patients into six separate groups: definite LNB, possible LNB, Viral CNS infection, non-\textit{Borrelia} Bacterial CNS infection, Other CNS disease (with pleocytosis) and Negative (without pleocytosis). A combined interpretation of four variables (leukocyte cell counts, protein concentration, CXCL13 and IL-6 concentrations in CSF) is presented using principal component cluster analysis. We included by chart review 390 patients discharged with definite LNB (n = 31), possible LNB (n = 10), confirmed Viral or non-\textit{Borrelia} Bacterial CNS infection (n = 34), Other CNS disease (n = 58), and Negative (n = 257) for CXCL13 and IL-6 analysis. Principal component analysis (PCA) revealed three distinct clusters based on leukocyte cell counts, protein concentration, CXCL13 and IL-6 concentrations in CSF from 380 included patients (10 possible LNB patients excluded). The clusters clearly differentiate the groups: definite LNB, non-\textit{Borrelia} Bacterial CNS infection and Negative (without pleocytosis). A receiver operating characteristic (ROC) curve comparing LNB patients (n = 31) and all non-LNB conditions with CSF pleocytosis (n = 99) indicated an optimal CXCL13 cut-off value of 50.7 pg/mL, resulting in a sensitivity and a specificity of 93.6 and 91.1%, respectively. The ROC analysis comparing patients with confirmed non-LNB CNS infection (n = 34) and all others with CSF pleocytosis (n = 97) resulted in an optimal IL-6 cut-off value of 111.5 pg/mL, yielding a sensitivity and a specificity of 78.8% and 82.5% respectively. Of the ten possible LNB patients, three cases (with CXCL13 levels above cut-off) fall within the LNB cluster, and one case is just outside, providing some laboratory support for the diagnosis of LNB. The remaining six possible LNB patients (with CXCL13 levels below the 50.7 cut-off) had little support for the diagnosis of LNB in the PCA-plot. The results of this study confirm that CXCL13 is a valuable supplement for diagnosis of LNB, and that the combination of CXCL13 and IL-6 may be used to differentiate cases of LNB from other CNS infections. Furthermore, IL-6 can be of differential diagnostic value when evaluating patients with possible LNB.

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1. Introduction

1.1. Diagnosing Lyme neuroborreliosis

Lyme borreliosis is a tick-transmitted disease caused by bacteria from the *Borrelia burgdorferi* sensu lato (Bb) complex (*Burgdorfer et al., 1982; Steere et al., 1983*). It is the most prevalent tick-borne infection in Western Europe with an increasing geographic distribution and annual incidence (*Sykes and Makiello, 2017*). Clinical symptoms of Lyme neuroborreliosis (LNB) often manifest as painful radiculitis and/or facial nerve palsy but can also be less characteristic and resemble neurological symptoms of other central nervous system (CNS) diseases. Examination of the cerebrospinal fluid (CSF) is essential for the diagnosis of LNB. A hallmark of LNB is mononuclear cell pleocytosis (> 4 cells/µL) together with elevated protein concentration (*Mygland et al., 2010; Stanek and Strle, 2018*). Laboratory diagnosis of LNB is presently made by calculating an antibody index (AI) based on intrathecal production of antibodies against Bb by comparing CSF and serum Bb antibody levels (*Hansen and Lebech, 1991; Strle and Stanek, 2009; Mygland et al., 2010; Stanek and Strle, 2018*). Almost all guidelines recommend CSF examination and detection of intrathecal antibody production for the diagnosis of LNB (*Eldin et al., 2019*). However, in the early stage of LNB, there are limitations to these antibody analyses because antibody production may be absent at a detectable level, making it difficult to discriminate LNB from other CNS diseases (*Jøstad and Mygland, 2008; van Burgel et al., 2011; Henkel et al., 2017*). The ESGOR guidelines specify that the diagnostic sensitivity of the intrathecal synthesis is about 80% in patients with shorter duration (< 6–8 weeks) of clinical disease and nearly 100% with longer disease duration (*Dessau et al., 2018*). Therefore, there is a risk that patients with a false-negative test result for intrathecal Bb-antibody synthesis will not receive the appropriate treatment with the risk of long-term sequelae of LNB. On the other hand, patients may be misclassified, based on vague clinical symptoms and a negative Bb-specific AI, and therefore receive unnecessary antibiotic therapy with the risk of consequences such as changes in the gut microbiome, allergic reactions, and colonization with antibiotic-resistant bacteria. Therefore, supplementary diagnostic tools in cases with a possible diagnosis of LNB are needed.

1.2. CXCL13 and IL-6

The C-X-C motif chemokine ligand 13 (CXCL13) is a protein produced and secreted by antigen-presenting cells such as dendritic cells and macrophages. CNS invasion by Bb species induces production of CXCL13, triggering lymphocyte migration to the location of infection, in turn leading to intrathecal production of Bb-specific antibodies (*Rupprecht et al., 2008*). In 2005 Rupprecht et al. suggested that CSF CXCL13 (hereafter CXCL13) could be used as a highly specific and sensitive diagnostic biomarker for LNB, especially in the very early stage of the disease course (*Rupprecht et al., 2005*). In an attempt to establish an international CXCL13 cut-off for LNB diagnostics, several studies have since demonstrated that the levels of CXCL13 were strongly associated with CSF pleocytosis and the presence of Bb-specific intrathecal produced antibodies. Augmented CXCL13 levels may also be seen in patients with a clinical LNB diagnoses but without positive Bb intrathecal AI results, implying a potential use of CXCL13 as biomarker in early LNB infections. However, these reports have also illustrated that in patients with possible/early LNB, the CXCL13 levels often overlap with other neuroinfections or neuroinflammatory conditions such as viral meningitis, neurosyphilis, CNS lymphoma, multiple sclerosis, and autoimmune encephalitis (*Rupprecht et al., 2005; Schmidt et al., 2011; Wagner et al., 2018; Pilz et al., 2020*). The incorporation of additional biomarkers in connection with CXCL13 may improve the discriminatory power in separating CNS infectious diseases. A potential candidate is the interleukin 6 (IL-6) pro-inflammatory cytokine that induces the expression of a variety of proteins responsible for acute-phase inflammation. High concentrations of IL-6 have been found in CSF samples from patients suffering from neuroinfections due to bacterial or viral etiology, but lower levels have been detected in CSF specimens from cases of LNB (*Cerar et al., 2013; Pietikäinen et al., 2016; Lennertiet al., 2019*).

1.3. Aim

The aim of this cross-sectional study was to measure the concentrations of CXCL13 and IL-6 in the CSF of patients examined for neuroinfections. This with the attempt to evaluate the diagnostic potential of these biomarkers alone and in combination with CSF cell count and protein concentration, in the differentiation between definite and possible LNB, as well as between definite LNB and other neuroinfections.

2. Materials and methods

2.1. Study design and CSF sample collection

This is a prospectively planned cross-sectional study including all patients examined for a central nervous system infection at Lillebaelt Hospital in the Region of Southern Denmark during April 2018 to November 2018, and April 2019 to September 2019. All CSF and blood samples were collected and primarily analysed as part of the routine diagnostics of patients with suspected central nervous system infection. Upon arrival at the Department of Clinical Microbiology at Lillebaelt Hospital, 250 µL CSF was pipetted and stored at −20 °C until cytokine analysis, which was routinely performed at the department once every second week. Furthermore, for verification of the immunoassay performance, 61 CSF aliquots (from June 2014 to February 2018) were selected from stored and previously examined CSF samples kept at −80 °C at the Department of Clinical Microbiology. Samples with enough material (≥ 250 µL CSF) were included based on I) a positive CSF/serum Bb-specific antibody index (as described below), or II) a positive polymerase chain reaction (PCR) for viruses or bacteria (as described below). Furthermore, a random selection of stored samples III), negative in both I and II, was also included. The 61 selected samples were used as controls for the CXCL13 and IL-6 analytical reactivity. These samples and patients were likewise included in the patient cohort of this project (Fig. 1).

2.2. Multiplexed fluorescent bead-based immunoassay

CSF samples were tested for CXCL13 and IL-6 simultaneously, using a duplex paramagnetic bead Bio-Plex Cytokine Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. A detailed description of the immunoassay procedure is presented in the supplemental material.

2.3. Evaluation of assay variance

To assess the intra- and inter coefficients of assay variation (CV) of the CXCL13 and IL-6 bead-based immunoassays, four quality controls consisting of cytokine standard (Cat. # 171DK0001, Bio-Rad) diluted in pooled CSF specimens negative for CNS infections (designated matrix diluent), were prepared. These dilutions were chosen so that most of the assay ranges were covered, i.e., high, medium, low, lower limit of quantification (LOLO).

2.4. Laboratory data

Data was extracted from the electronic local laboratory data management systems for clinical biochemistry (BCC) and microbiology (MADS). The routine procedures for CSF leukocyte cell counts and measurement of CSF protein concentration were applied to all CSF samples. Serum and CSF were analysed by relevant microbiological...
Fig. 1. Inclusion and grouping of patients. A) flowchart of the inclusion and classification of 390 patients. B) Box chart illustrating the relative distribution of the 61 retrospectively selected and 329 prospectively collected patient samples included.
laboratory tests (list below) based on patients’ neurological symptoms at the time of lumbar puncture.

- CSF/serum Bb-specific antibody index (AI) (IDELA Lyme Neuroborreliosis test, Oxoid, Hampshire, UK) (Hansen and Lebecch, 1991)
- Bacterial culture of CSF
- CSF polymerase chain reaction (PCR) for viruses (Cytomegalovirus (CMV), Enterovirus (EV), Herpes simplex virus 1 (HSV-1), Herpes simplex virus 2 (HSV-2), Human herpesvirus 6 (HHV-6), Human parechovirus (HPeV) and Varicella zoster virus (VZV)) and bacteria (E. coli K1, H. influenzae, L. monocytogenes, N. meningitidis, S. agalactiae and S. pneumoniae) by FilmArray Meningitis/Encephalitis (ME) Panel (BioFire Diagnostics, Salt Lake City, Utah, USA)
- Serology and CSF PCR for TBE virus, and measurement of intrathecal HSV-1, HSV-2 or VZV antibodies were done at the Statens Serum Institut (Copenhagen, Denmark)

2.5. Clinical data and classification of patients

In the present study, all 390 patients included were grouped based on predefined classification criteria using the observational data and the discharge diagnosis from the electronic medical record. If recorded, the clinical data included age, sex, date of symptom onset, neurological symptoms, duration of neurological symptoms, tick exposure, recollection of tick bite and/or erythema migrans, antimicrobial treatment prior to lumbar puncture, antibiotic regimen after spinal tap and discharge diagnosis code (ICD-10) at discharge or at follow-up consultations. The clinical discharge diagnoses were determined by physicians examining the patients in the clinic, independently of this study.

- Definite LNB. Definite diagnosis of LNB were made, when patients fulfilled all three diagnostic criteria for LNB according to the EFNS guidelines (Mygland et al., 2010), i.e. clinical symptoms consistent with LNB and absence of other possible causes, CSF pleocytosis (CSF leukocyte cell counts ≥ 5 cells/µL) and evidence of intrathecal produced Bb IgM and/or IgG by a positive Bb AI
- Possible LNB. Clinical discharge diagnosis, when two out of three EFNS diagnostic criteria is present, and especially by the patient showing clinical response during antibiotic treatment (resolution of clinical symptoms described in the electronic medical record of the patient)
- Viral CNS infection. Confirmed CNS infections identified by CSF PCR of known pathogenic viruses
- Bacterial CNS infection. Confirmed CNS infections identified by culture or PCR of known non-Borrelia burgdorferi sensu lato bacteria in CSF
- Other CNS disease. Patients with CSF pleocytosis and no infectious etiology documented
- Negative. Patients without CSF pleocytosis (CSF leukocyte cell counts < 5 cells/µL) and no infectious etiology documented

2.6. Statistics

CXCL13 and IL-6 results below the lower limit of quantification (LLOQ) were assigned values of half the lower limit and values above the upper limit of quantification (ULOQ) were assigned values of double the upper limit. Numerical variables describing patient characteristics (age, CSF leukocyte cell counts, CSF protein concentration, and CXCL13 and IL-6 concentrations) were not normally distributed and therefore sum of both assays and therefore we continued using undiluted patient specimens. A total number of 390 CSF specimens were thus included and samples from 23 patients were not analysed due to insufficient sample material.

3. Results

3.1. Verification of assay performance

The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) of the CXCL13 and IL-6 immunoassays were 0.35 and 5578 pg/mL, and 0.88 and 12,132 pg/mL, respectively. All 61 retrospectively selected CSF samples were within the LLOQ and ULOQ range of both assays and therefore we continued using undiluted patient specimens. Among the 390 patient samples, one sample was above the ULOQ, and 34 samples were below the LLOQ of the CXCL13 assay. Five samples were above the ULOQ, and 21 samples were below the LLOQ of the IL-6 assay. The intra- and inter-assay coefficients of variation (%CV) ranged from 4.2% to 9.9% and from 13.6% to 29.0%, for the CXCL13 assay, and between 2.4% to 6.1%, and 9.0% and 12.2% for the IL-6 assay, respectively (Table S1-S4). The overall accuracy and precision of the CXCL13 and IL-6 immunoassays are further detailed in the Supplemental Tables S1-S4.

3.2. Patient inclusion and characteristics

During the inclusion period, the Department of Clinical Microbiology at Lillebaelt Hospital received CSF for microbiological testing from 352 patients. However, samples from 23 patients were not analysed due to blood contamination of CSF (n = 8), not enough sample material (n = 12) or samples had not been stored at the correct temperature (n = 3). Four patients received more than one lumbar puncture, but only the cytokine results from the first puncture were included in this dataset. Thus, 329 patients were prospectively included in this study and together with previously collected and stored sample material from 61 patients, a total number of 390 CSF specimens were thus included and analysed for CXCL13 and IL-6 levels as summarized in Fig. 1. There were 53% women, and the median age was 46 years (range 0–91 years) (Table 1). The number of children (< 18 years) included was 51, constituting 13.1% of the patients. Median leukocyte cell counts,
together with median protein, CXCL13 and IL-6 concentrations of different diagnostic groups are shown in Table 1, and the CXCL13 and IL-6 quantities are presented in Fig. 2 A and B respectively. Among the 390 patients included, 31 (7.9%) were diagnosed with definite LNB (10 were below the age of 18) and 34 (8.7%) with a specific neuroinfection other than LNB. Among the 31 patients with definite LNB, 17 (54.8%) were positive in both IgM and IgG Bb AI, whereas six (19.4%) and eight (25.8%) were only positive in IgM or IgG, respectively. The possible LNB group consists of 10 (2.6%) patients with clinical symptoms of LNB, eight with CSF pleocytosis, hence given a clinical LNB diagnosis, and two without CSF pleocytosis but with a positive Bb AI, all 10 patients thus fulfilling 2 out of 3 EFNS criteria as summarized in Table 2. Children constitute 5 out of 10 patients in the possible LNB group.

### 3.3. Separation of diagnostic groups by principal component analysis

Principal component analysis (PCA) defines artificial variables (principal components) that explain as much of the variation in the data as possible. Therefore, the PCA will summarize the information of multiple correlated variables in principal components, which can be

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics and laboratory results of all 390 included patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sex</td>
</tr>
<tr>
<td></td>
<td>(F/M)</td>
</tr>
<tr>
<td>definite LNB</td>
<td>14/17</td>
</tr>
<tr>
<td>(n = 31)</td>
<td></td>
</tr>
<tr>
<td>Possible LNB</td>
<td>3/7</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>Viral CNS infection*</td>
<td>14/14</td>
</tr>
<tr>
<td>(n = 28)</td>
<td></td>
</tr>
<tr>
<td>Non-Bb Bacterial CNS infection#</td>
<td>4/2</td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>Other CNS disease</td>
<td>30/28</td>
</tr>
<tr>
<td>(n = 58)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>143/114</td>
</tr>
<tr>
<td>(n = 257)</td>
<td></td>
</tr>
</tbody>
</table>

* The viral CNS infections were HSV-1 (n = 2), HSV-2 (n = 4), VZV (n = 7), HHV-6 (n = 3), Enterovirus (n = 11), TBE virus (n = 1). To be noted, one enterovirus case was without pleocytosis.

# The non-Bb bacterial CNS infections were S. pneumoniae (n = 4), E. coli (n = 1) and H. influenzae (n = 1). Bb = Borrelia burgdorferi sensu lato.

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Fig. 2. Cerebrospinal fluid CXCL13 and IL-6 concentrations. CXCL13 (A) and IL-6 (B) levels in different diagnostic groups with medians and 95% confidence intervals. A) The dashed red line indicates the calculated optimal CXCL13 cut-off value of 50.7 pg/mL. B) The dashed red line indicates the optimal IL-6 cut-off value of 111.5 pg/mL.
used to visualize general trends in the dataset using a 2-dimensional scatterplot. The PCA was performed on data from 380 patients (possible LNB cases were excluded) and show (Fig. 3) that the two novel principal components explain 83.1% (Scree plot Figure S1) of the variance of variables CXCL13, IL-6, protein concentration and CSF leukocyte cell counts. The scree plot and the factor map of the PCA are shown in Table 2.

Table 2
Discharge diagnosis, clinical and laboratory findings in possible LNB group.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age</th>
<th>Neurological symptomsa</th>
<th>Observed tick bite/erythema migransa</th>
<th>Duration of symptomsb</th>
<th>CSF leukocyte cell counts (%) (mononuclear cells)</th>
<th>Bb AI</th>
<th>CXCL13 (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>Discharge diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical symptoms, CSF pleocytosis and CXCL13 below cut-off</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>Peripheral nerve palsy, headache, fatigue</td>
<td>No</td>
<td>3 days</td>
<td>22 (95.4%)</td>
<td>Negative</td>
<td>0.2</td>
<td>533</td>
<td>Lyme disease</td>
</tr>
<tr>
<td>42</td>
<td>51</td>
<td>Radicular pain, headache</td>
<td>No</td>
<td>7 days</td>
<td>171 (97.1%)</td>
<td>Negative</td>
<td>11</td>
<td>8.8</td>
<td>Borrelia polyradiculitis</td>
</tr>
<tr>
<td>89</td>
<td>69</td>
<td>Radicular pain, Peripheral nerve palsy</td>
<td>No</td>
<td>3 days</td>
<td>11 (not determined)</td>
<td>Negative</td>
<td>15.9</td>
<td>10.6</td>
<td>Borrelia polyradiculitis</td>
</tr>
<tr>
<td>92</td>
<td>4</td>
<td>Peripheral nerve palsy</td>
<td>Yes/no</td>
<td>2 days</td>
<td>14 (not determined)</td>
<td>Negative</td>
<td>17</td>
<td>2.5</td>
<td>Lyme disease</td>
</tr>
<tr>
<td>107</td>
<td>29</td>
<td>Fever, abducens nerve palsy</td>
<td>Yes/no</td>
<td>4 days</td>
<td>170 (76.5%)</td>
<td>Negative</td>
<td>11.5</td>
<td>764.2</td>
<td>Borrelia polyradiculitis</td>
</tr>
<tr>
<td>Clinical symptoms, CSF pleocytosis and CXCL13 above cut-off</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>171</td>
<td>4</td>
<td>Peripheral nerve palsy, fatigue</td>
<td>Yes</td>
<td>1 day</td>
<td>110 (100%)</td>
<td>Negative</td>
<td>199.3</td>
<td>52.3</td>
<td>Lyme disease</td>
</tr>
<tr>
<td>323</td>
<td>5</td>
<td>Peripheral nerve palsy, headache</td>
<td>Yes/no</td>
<td>1 day</td>
<td>190 (94.7%)</td>
<td>Negative</td>
<td>314.4</td>
<td>223.8</td>
<td>Lyme disease</td>
</tr>
<tr>
<td>341</td>
<td>68</td>
<td>Peripheral nerve palsy, radicular pain</td>
<td>No</td>
<td>17 days</td>
<td>85 (100%)</td>
<td>Negative</td>
<td>125.4</td>
<td>2.3</td>
<td>Lyme disease</td>
</tr>
<tr>
<td>Clinical symptoms and positive Borrelia AI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>39</td>
<td>Peripheral nerve palsy, fatigue</td>
<td>Yes</td>
<td>27 days</td>
<td>4 IgM/IgG positive</td>
<td>2.1</td>
<td>1.5</td>
<td>Symptoma systematis nervosa + Lyme disease</td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>6</td>
<td>Fatigue, headache</td>
<td>No</td>
<td>315 days</td>
<td>2 IgG positive</td>
<td>5.5</td>
<td>2.1</td>
<td>Lyme disease</td>
<td></td>
</tr>
</tbody>
</table>

a At time of lumbar puncture. b < 6 months before lumbar puncture. c CSF leukocytes x 10⁹/L. Bb AI = Borrelia specific IgM/IgG intrathecal antibody index.

Fig. 3. Principal component analysis. PCA-plot visualizing the principal component scores and loading vectors of variables; CXCL13, IL-6, CSF protein concentration, and leukocyte cell counts on 380 patient cases. The plot shows the observations as points in the 2D-scatterplot formed by the principal components PC1 and PC2. The ellipses show the 95% limits of the multivariate normal distribution around the cluster mean (shown as larger group symbol). The length of the arrow represents the quality/strength of the variable (A random variable would have a short arrow in a random direction). The direction of the arrows indicates how closely the variables are grouped together. Dark blue dots equal possible LNB cases. The PCA cluster analysis was performed in R software using packages “FactoMineR” and “factoextra”.

6
supplemental Figure S1. Fig. 3 shows the correlation arrows for the four variables (CXCL13, IL-6, protein concentration, and CSF leukocyte cell counts). The principal component 1 (PC1) accounts for 66.4% of the variance (Scree plot Figure S1) and is almost equally based on the four variables (factor map Figure S1) with the leukocyte cell count being dominant. The correlation arrows of the variables have only positive PC1 coordinates indicating a common data trend. The PC2 accounts for 16.7% of the variance and is unlike PC1 mostly based on CXCL13 and IL-6 (factor map Figure S1). On the PC2 scale, CXCL13 and IL-6 correlation arrows are clearly separated as indicated by a > 90° angle between them, and is therefore negatively correlated, thus representing two different clusters (Fig. 3). Increased leukocyte cell counts together with the protein concentration are general features as indicated by the horizontal direction of the arrows and are therefore not accountable for cluster separation on the PC2 scale. Increased CXCL13 quite clearly separates a cluster of patients with definite LNB. Increased IL-6 levels in CSF together with a high leukocyte cell count differentiates the non-Bb Bacterial CNS infection group from the other groups. In addition, viral CNS infection has IL-6 reactivity in some patients. The possible LNB cases (dark blue dots in Fig. 3) are not included in the PCA model but predicted from the model using the PCA information and parameters obtained with the 380 patients. Of the 10 cases with possible LNB, three cases fall within the LNB ellipse (IDs 171, 323, 341) and one just outside (ID 42), indicating some laboratory support for diagnosis of LNB in these four cases. However, the remaining six cases have little support for the diagnosis of LNB, because four fall within the negative group (IDs 29, 89, 92, 138) and the last two cases (ID 11 and ID 107) are in the viral cluster in the upper left quadrant.

The cases in the possible LNB group were further evaluated, based on the PCA analysis only. In Fig. 4 and Table 3 are shown the 10 cases with possible LNB. The probability (in%) of each possible LNB case of belonging to each cluster mean was calculated. The only case with a probability of belonging to the LNB cluster is case ID 341 with a probability of 10.8%. Case no. 42, 171 and 323 have a low probability (0.7–2%) of belonging to the LNB cluster and other diagnoses are more likely. The case 171 highlighted as an example in both the table and the figure could be LNB, other CNS disease or viral infection with probabilities of 1.9%, 7.0% and 5.7%, respectively.

3.4. CXCL13 cut-off

Receiver operating characteristic (ROC) curve plotted for the performance of discrimination between definite LNB patients (n = 31) and all non-LNB conditions with CSF pleocytosis (n = 99) resulted in an area under curve (AUC) of 0.976 (Fig. 5). At cut-off value of 8.26 pg/mL, the sensitivity and specificity were 100% and 71.2%, respectively. At cut-off value of 496.6 pg/mL, the sensitivity and specificity were 83.9% and 100%, respectively. The calculated highest Youden Index score was 0.85 at CXCL13 cut-off 50.7 pg/mL, resulting in an optimal balance between a sensitivity and a specificity of 93.6 and 91.1% respectively. When including all patients (n = 390) in the ROC-analysis the AUC increased to 0.993. The 50.7 pg/mL CXCL13 cut-off still resulted in the highest Youden Index score and a sensitivity of 93.6% and a specificity of 97.5%.

Table 3

<table>
<thead>
<tr>
<th>ID</th>
<th>possible LNB</th>
<th>Non-Bb Bacterial CNS infection</th>
<th>definite LNB</th>
<th>Negative</th>
<th>Other CNS disease</th>
<th>Viral CNS infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
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Fig. 4. Further analysis of the cases with possible LNB. Plot based on the first and second dimension of the principal component analysis with the means of each of the five clusters for the diagnostic groups. As an example, case with ID 171 is shown with lines to each cluster mean. Each case is labeled with a case ID.
3.5. IL-6 cut-off

ROC plotted for the performance of classification between patients with confirmed non-LNB CNS infection (n = 34) and all other patients with CSF pleocytosis (n = 97) resulted in an AUC of 0.869 (Fig. 6). At cut-off value of 4.35 pg/mL, the sensitivity and specificity were 100% and 35.1%, respectively. At cut-off value of 987.7 pg/mL, the sensitivity and specificity were 51.5% and 100%, respectively. The calculated highest Youden Index score was 0.61, at IL-6 cut-off 111.5 pg/mL, resulting a sensitivity and a specificity of 78.8% and 82.5% respectively. When including all the patients in the ROC-analysis the AUC increased to 0.925. The optimal cut-off value based on the calculated highest Youden Index of 0.66 increased to 119 pg/mL IL-6 with a sensitivity of 73.5% and a specificity of 92.7%.

3.6. CXCL13 and IL-6 results in the patient cohort

Ninety-three percent of patients (29/31) in the definite LNB group had a CXCL13 result above the CXCL13 cut-off of 50.7 pg/mL, while 30% of patients (3/10) in the possible LNB group had CXCL13 levels above 50.7 pg/mL (Fig. 2A). Two patients with a confirmed CNS infection, both with a non-Bb bacterial etiology, had CXCL13 results above cut-off. Four patients in the Other CNS Disease group had CXCL13 levels above the CXCL13 cut-off (Table 4). In seventy-six percent of CSF from patients (26/34) with confirmed CNS infections, the IL-6 result was above the IL-6 cut-off of 111.5 pg/mL (Fig. 2B). All the IL-6 results from
Patients with non-Bb bacterial etiologies were far above the IL-6 cut-off, but eight patients with CNS infection of viral etiology (3/7 VZV, 2/11 Enterovirus and 3/3 HHV6) did not have elevated IL-6 levels above cut-off. Four of 31 definite LNB patients had IL-6 levels above cut-off, while three of 10 possible LNB patients had IL-6 levels above cut-off. Among patients with CSF pleocytosis but without a confirmed infectious etiology, 10/58 (17%) had IL-6 levels above cut-off, while only 3% of patients from the negative group (9/257) had IL-6 levels above cut-off.

4. Discussion

In this study, we evaluated a duplex bead-based immunoassay for simultaneous quantification of CXCL13 and IL-6 in CSF specimen aliquots from patients examined for a central nervous system infection in the clinical setting of our hospital. Our results support the role of CXCL13 in CSF as diagnostic biomarker of LNB and that IL-6 can be of extra value as a differential biomarker when evaluating patients with possible LNB or other types of neuroinfections.

Principal component analysis was used as a method for exploratory data analysis, to get a statistical impression of four inter-correlated quantitative variables of cerebrospinal fluid analysis (CXCL13, IL-6, protein, and leukocyte cell counts), and to visually inspect whether the different diagnostic groups were to be found in distinct clusters and how well they were separated. The PCA biplot in Fig. 3 shows how the four variables are correlated and the resulting principal components plotted in a two-dimensional graph. The PCA calculation does not include prior knowledge of the diagnostic groups. They are only added as means of points and the ellipses in the plot, all marked with different colours. Thus, it is the numerical data that suggest the existence of meaningful clusters. The diagnostic groups are just added to see if these appear consistent with the “natural” distribution of the data points. As shown in Fig. 3, the PCA suggests three distinct clusters (negative cases, LNB and non-Bb Bacterial CNS infection). Whereas Other CNS diseases and Viral CNS infections does appear to have a more diffuse distribution, albeit mostly in the direction of the IL-6 dimension. The principal component PC1 is almost equally based on the four variables used (factor map, Figure S1), thus PC1 demonstrates the presence of CNS inflammation and differentiate the non-inflammatory negative cases from the rest. The principal component PC2 separates the LNB group from the non-Bb Bacterial CNS diseases well and is largely based on IL-6 and CXCL13 (factor map, Figure S1). The model presented here could be used as support to the clinical decision as illustrated for the ten cases of possible LNB showing that four falls into or close to the definite LNB cases, whereas in six cases other diagnoses should be considered. This analysis does not provide a clear and final diagnosis but gives an indication of how much the pattern of the results of leukocyte cell counts, CSF protein concentration, the CSF levels of IL-6 and CXCL13 may support a diagnosis of LNB or other CNS infections. Thus, supporting a clinical decision of treatment and further diagnostic work up. For such a cluster model to be applicable for clinical decision support in general, it would be important that data from other clinical centres would yield similar cluster tendencies. This should in theory be independent of the numerical distribution of diagnoses and case mix, only the number of data points in each cluster should differ. What could differ is the distribution of cases with variations in diagnostic assignment due to difference in case definitions and clinical practice. Studies have been published showing similar clearly separated clusters of LNB cases from other neurological disorders by use of CXCL13 and leukocyte cell counts (Markowicz et al., 2018; Eckman et al., 2021). In the same manner, a recent study showed that a combination of CXCL13 and CCL19 in CSF from children could be used to classify probable LNB cases as LNB (Barstad et al., 2020). There is an increasing tendency, that the diagnostic industry provides automated devices to measure a larger panel of biomarkers targeting for example CNS disease. The cluster analysis presented here could be a useful tool to assist in diagnostic interpretation of such multiple biomarker results.

The optimal CXCL13 cut-off value for classification between definite LNB and patients with CSF pleocytosis was calculated by a ROC analysis to be 50.7 pg/mL. When using this CXCL13 cut-off, two definite LNB cases had a CXCL13 result below the cut-off (8.3 and 28.3 pg/mL) (Fig. 2). Notably, one of the LNB cases with a CXCL13 result below CXCL13 cut-off was administered amoxicillin and prednisolone prior to lumbar puncture. The two definite LNB cases with CXCL13 results below cut-off had peripheral nerve palsy and a short period of symptoms (< 7days) before lumbar punctures were performed. Antimicrobial treatment and/or short duration of symptoms prior to lumbar puncture may explain the relatively low amounts of CXCL13 quantified, as stated by others (Schmidt et al., 2011; Bremell et al., 2013; Knudtzen et al., 2020). Three possible LNB cases had CXCL13 levels above the optimal CXCL13 cut-off of 50.7 pg/mL, which strongly supports the clinical discharge diagnoses of Lyme borreliosis. From the Other CNS disease group four patients had CXCL13 results above cut-off, however all four patients were diagnosed with non-CNS infectious diseases at discharge (Table 4).

The optimal CXCL13 cut-off value found in this study is low compared to other studies using bead-based immunoassays for CXCL13 quantification with cut-off ranging between 131 pg/mL to 1726 pg/mL (Markowicz et al., 2018; Barstad et al., 2020; Eckman et al., 2021) van Gorkom et al., 2021). However, our optimal cut-off is in accordance with a Danish retrospective study in which the optimal CXCL13 cut-off was determined to be 49 pg/mL using a CXCL13 ELISA assay (Knudtzen et al., 2020). Large discrepancies between CXCL13 cut-offs, due to e.g., heterogeneity between study populations and CXCL13 assay choice, has made it difficult to establish an international consensus cut-off value. A meta-analysis published by Rupprecht et al. found that, for cross-sectional studies only, a CXCL13 cut-off value of 91 pg/mL was optimal for discrimination between patients with LNB and patients with other neurological disorders (Rupprecht et al., 2018). Interestingly, our next best CXCL13 cut-off value based on the Youden Index was at 102.1 pg/mL, which is relatively close to the optimal cut-off for cross-sectional studies from the meta-analysis. When using our next best CXCL13 cut-off, one additional definite LNB patient had CXCL13 levels below cut-off in our study. However, one patient with Other CNS disease had a CXCL13 below cut-off (ID - 287, Table 4), thereby marginally increasing the diagnostic specificity.

ROC-plots were also made to estimate the diagnostic sensitivity and specificity of the IL-6 immunoassay in discrimination between patients
with a non-LNB neuroinfection and patients with CSF pleocytosis. At optimal IL-6 cut-off value of 111.5 pg/mL, the sensitivity and specificity were calculated to be 78.8% and 82.5% respectively. In our study, all patients with non-Bb Bacterial CNS infection had high IL-6 concentrations in CSF (Table 1), thus IL-6 is a potential biomarker of neuroinfections due to e.g., *S. pneumoniae*, *E. coli* and *H. influenzae*. A study recently demonstrated compatible classification power of IL-6 in bacterial neuroinfections as found in our study (Kalchev et al., 2021). Eight patients with CNS infection of viral etiology did not have IL-6 levels above cut-off. Interestingly, all patients with positive HHV-6 PCR results did not have elevated IL-6 concentrations. This indicates that HHV-6 CNS infections have a different biochemical profile based on a distinct neuropathology compared to other neuroinfections and may merely represent an asymptomatic reactivation of HHV-6 from a previous infection. IL-6 is an acute-phase protein that is produced in response to infections and tissue injuries and is therefore, like CXCL13, not specific for CNS diseases with microbial etiology. However, in our study, seventy-six percent of patients with a confirmed non-LNB CNS infection did have elevated IL-6 levels in CSF, while only three percent of patients without CSF pleocytosis had augmented IL-6 levels. This result supports that high levels of IL-6 in CSF can be used as a biomarker of CNS infection. Furthermore, it could be interesting to use next generation sequencing techniques on samples with augmented IL-6 levels and CSF pleocytosis to explore unidentified microorganism causing potential CNS infections.

CXCL13 and IL-6 can be used as differential diagnostic markers, but they cannot stand alone as described by the numerous exceptions in our study. Therefore, the LNB diagnosis is still based on a constellation of clinical and paraclinical findings.

The analytical robustness of the CXCL13 and IL-6 immunoassays was also assessed in our study. When examining the stability of the CXCL13 and IL-6 assays using the manufacturer’s standards, the inter-assay variations are less than 15% for both assays. An inter-assay%CV less than 15% is often used as benchmark for reliable assay performance. However, when using an in-house control, the%CV is much greater. This increase in variation is likely due to some decay of CXCL13 and IL-6 proteins in the in-house control. An international CXCL13reference standard that could be applied as both calibrator between assays and as quality control is therefore highly desirable.

The main strengths of our study are the study design and the simultaneous quantification of cytokines. This large cross-sectional study, consecutively including patients examined for CNS infections, both with and without pleocytosis thereby minimizing selection bias. Our patient cohort included patients, both children and adults, with a broad range of diagnoses other than LNB. Aliquots of routine CSF specimens were made specifically for this study. The duplex bead-based immunoassay made it possible to quantify the CXCL13 and IL-6 concentrations in parallel, thus preventing additional freeze-thaw cycles, which could have affected CXCL13 and IL-6 results, due to reduced protein quality.

This study has some limitations. One limitation is that the group of possible LNB patients is relatively small making it difficult to generalize our findings. Therefore, the combination of CXCL13 and IL-6 needs to be evaluated further in future studies with larger cohorts, including more possible LNB cases, to better estimate the usefulness and diagnostic accuracy of these biomarkers. Another limitation is that the possible LNB patients did not have a second lumbar puncture performed, which could have supported the clinical discharge diagnosis of LNB, especially if the *Borreli* etiology could be corroborated by intrathecal antibody production or by *Borreli*-specific PCR. In addition, a drawback is that the electronic medical record system applied by our hospital is primarily based on narrative text that lack formal standardization regarding collection of anamnestic and clinical information, which made it difficult to determine tick exposure, recollection of tick bite and/or erythema migrans and antimicrobial treatment prior to lumbar puncture for all patients. Furthermore, it would have been very interesting to evaluate patients diagnosed with neurosyphilis or CNS lymphoproliferative disorders in this study, as these are important differential diagnosis regarding CXCL13. However, both neurosyphilis and CNS lymphoma are rare diagnoses in our region and did not occur in our patient cohort during the study period.

5. Conclusion

In conclusion, this study supports the role of CXCL13 in CSF as diagnostic biomarker of LNB. Our results also show that IL-6 can be of additional value as a differential diagnostic marker when evaluating patients with possible LNB. Whether the addition of both CXCL13 and IL-6 to the paraclinical analysis on CSF will improve the diagnostics of patients with possible LNB beyond the diagnostic value of CXCL13 alone, remains, of course, to be further evaluated in new studies. However, the clear dispersion of the possible LNB cases in our study suggest that other diagnoses should also be looked for instead of LNB.

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CRediT authorship contribution statement

Trine Andreaesen Leth: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Visualization, Writing – original draft. Ram Benny Dessau: Formal analysis, Methodology, Supervision, Visualization, Writing – original draft. Jens Kjelseth Møller: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft.

Declaration of Competing Interest

Trine Andreaesen Leth and Jens Kjelseth Møller declare no conflicts of interest. Ram B. Dessau declares the following financial interests/personal relationships which may be considered as potential competing interests: Participation in Advisory Board Meeting with Roche Diagnostics in 2018.

Data availability

The dataset is available, from the corresponding author upon reasonable request, in an anonymized form.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2022.101984.

References
