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Diagnostic performance of cerebrospinal fluid chemokine CXCL13 and antibodies to the C6-peptide in Lyme neuroborreliosis.

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Introduction

Lyme borreliosis (LB) is the most common tick-borne infection in both Europe and North America. It is caused by the spirochetes of the *Borrelia burgdorferi* sensu lato complex (1-2). The disease may present with symptoms from many different organs, of which Lyme neuroborreliosis (LNB) is the most common manifestation of disseminated borreliosis in Europe (3). Diagnosis of LNB relies on neurological symptoms and signs such as facial palsy, other cranial neuritis, radiculoneuropathies and meningitis, supported by laboratory findings of borrelia-specific intrathecal antibodies and pleocytosis in cerebrospinal fluid (CSF) (4). However, pleocytosis may be absent and specific intrathecal antibodies expressed as anti-borrelia antibody index (AI) in paired CSF/serum samples may be negative the first few weeks, and in addition, a positive AI may also persist several months and even years despite appropriate antibiotic treatment (5-11). Therefore, there is a need for additional markers of ongoing LNB. In recent years, the B-lymphocyte attracting chemokine CXCL13 has been proposed as an early CSF marker for LNB (12-14), and the commercial C6 antibody test has also been evaluated as a marker for LNB in CSF, although with inconsistent results (15-16). In this study, we aimed at further evaluating C6 antibody and CXCL13 results separately and in combination as markers for LNB. To achieve this, a large material of collected CSF/serum samples from patients (n=261) was tested and also analysed for albumin and total concentrations of IgM and IgG to allow comparisons and calculations of ratios and indices.

Patients and Methods

Patients and samples

The study was approved by the Regional Ethical Review Board in Linköping, Sweden. From the laboratory database at the Department of Clinical Microbiology, Kalmar County Hospital, a total of 124 AI positive patients (using IDEIA Lyme Neuroborreliosis, K6028, Dako Cytomation, UK) patients were identified and matched to 124 AI negative cases as previously described (17). From the neighbouring County of Jönköping, an additional 90 patients with intrathecal anti-borrelia antibodies (ITA) using the same test antigen; flagellum (Lyme Borreliosis ELISA kit 2nd Generation, Dako Cytomation, A/S, Glostrup, Denmark), could be identified from the same years, 2003-2005, and were included together with 25 ITA negative patients. While AI compares ratios of specific anti-borrelia antibodies in paired CSF/serum samples, ITA is based on the CSF sample only. According to the manufacturer, cut off levels for ITA have been chosen considerably above optical density (OD) values generally found when analysing CSF from patients with meningeal inflammation not due to LNB in order to avoid false positive results due to leakage of serum antibodies trough a disturbed blood-brain-barrier. AI/ITA will hence forth be collectively termed CSF anti-borrelia antibodies (CSF ABA).

The lumbar puncture was performed because of a suspected LNB, and all samples had been examined at the Department of Clinical Microbiology in Jönköping or Kalmar, respectively. Serum samples from patients in Kalmar County were investigated with Immunetics Quick ELISA Borrelia C6 Assay kit, Immunetics, Cambridge, MA, USA, and serum samples from patients in Jönköping with Lyme Borreliosis ELISA kit 2nd Generation, Dako Cytomation, A/S, Glostrup, Denmark.

Sufficient CSF/serum sample material was found in 261 of a total material of 363 patients. The incomplete samples from the missing 102 patients originated from 57 patients who were

CSF ABA positive with pleocytosis ($\geq 6*10^6$ leukocytes/L CSF), 25 patients who were either CSF ABA positive or with pleocytosis and finally 20 patients who were CSF ABA negative without pleocytosis. Thus, in this study 261 patients were investigated. CSF cell counts and medical records were examined for all patients and sex, age, symptoms and duration of symptoms were registered. The patients were then divided into four groups based on pleocytosis and CSF ABA results, see Table 1.

Laboratory analyses

Samples had been stored for 3-6 years (median 4.3) in –20° C until analysis. The chemokine CXCL13 was measured in serum and CSF by ELISA (Quantikine, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. In order to save material for multiple analyses, samples with results > 500 pg/mL were not diluted to obtain a final concentration and were therefore assigned the value 500 pg/mL. All samples were then refrozen before analyses of albumin and total concentrations of IgM and IgG in serum and CSF (rate nephelometry using Beckman Coulter Immage 800) and then refrozen again. Finally, C6 antibodies (Immunetics C6 Lyme ELISA Kit, Cat. No.: DK-E601-096; C6 test) were analysed in CSF by using the manufacturer's instructions for serum samples (dilution 1:20) as there is no formal recommendation regarding CSF analysis. Skarpaas et al.(15) used undiluted CSF for measurements of C6 antibodies, and accordingly, we also performed additional C6 testing using undiluted CSF in the still available samples (n=233). All analyses were performed without knowledge of any other results. Values with undetected levels were assigned half the value of the lower detection limit. The lowest detection limits in CSF were: albumin 0.10 g/L, IgM 0.3 mg/L and IgG 10 mg/L. The lowest detection limit for CXCL13 in both CSF and serum was 7.8 pg/mL. The inter-assay coefficient of variation (CV) was 3.2/5.3% for Serum-/CSF-albumin, 3.9/2.3% for Serum-/CSF-IgM and 3.7/5.2% for Serum-

Table 1. Patient characteristics at diagnostic lumbar puncture in 261 cases investigated for suspected Lyme neuroborreliosis.

	Group 1 *n=124*	Group 2 *n=19*	Group 3 *n=26*	Group 4 *n=92*
CSF Anti-borrelia antibodies[a]	+	+	−	−
CSF Pleocytosis	+	−	+	−
Clinical interpretation according to EFNS guidelines	Definite LNB	Possible LNB		Non-LNB
Men	75	14	19	49
(%)	(60)	(74)	(73)	(53)
Women	49	5	7	43
(%)	(40)	(26)	(27)	(47)
Median age (years)	37.5	55	8	47.5
range	(3-87)	(18-76)	(2-62)	(2-83)
Head/neck pain	67	5	12	42
(%)	(54)	(26)	(46)	(46)
Cranial nerve palsy	64	3	17	13
(%)	(52)	(16)	(65)	(14)
Radiculitits	43	6	2	10
(%)	(35)	(32)	(7.7)	(11)
Other symptom	13	8	2	34
(%)	(10)	(42)	(7.7)	(37)
Median duration of symptoms before LP (weeks)[b]	3.0[b]	4.0	1.0[b]	4.0[b]
range	(0-32)	(0.1-77)	(0.1-8.6)	(0-730)

n = Numbers.
CSF = Cerebrospinal fluid.
[a] Either borrelia-specific antibody index or intrathecal anti-borrelia antibodies.
Pleocytosis = Total white blood cell count ≥ $6*10^6$/L CSF.
EFNS = European Federation of Neurological Societies.
LNB = Lyme neuroborreliosis.
[b] Data missing on 1 patient in group 1, 1 in group 3 and 15 in group 4.
Patients could have one or more of the symptoms head/neck pain, cranial nerve palsy and radiculitis.
Patients with none of the above symptoms were classified as “other symptom”.

/CSF-IgG. The intra-assay CV was 2.8/3.2% for Serum-/CSF-albumin, 4.3/4.7% for Serum-/CSF-IgM and 4.5/0.6% for Serum-/CSF-IgG. The precision data were determined at the Department of Clinical Chemistry, Kalmar County Hospital. The inter-assay CV was 9.6% for CXCL13 and 11.5% for the C6 test and the intra-assay CV 4.3% and 10.1%, respectively, according to the manufacturer. Although a strict precision study was not performed for CXCL13, one internal control at approximately 50 pg/mL was run at three separate locations per 96 well microplate. Based on results from 15 microplates, an intra-assay CV of 9.0% and an inter-assay CV of 15.3% was found.

Calculations of intrathecal synthesis

The following formulas were used in order to calculate ratios and indices:

$$\text{Albumin ratio} = \frac{\text{CSF - albumin (mg/L)}}{\text{Serum - albumin (g/L)}}$$

$$\text{Total-IgM index} = \frac{\text{CSF - IgM (mg/L)} \,/\, \text{Serum - IgM (g/L)}}{\text{CSF - albumin (mg/L)} \,/\, \text{Serum - albumin (g/L)}}$$

$$\text{Total-IgG index} = \frac{\text{CSF - IgG (mg/L)} \,/\, \text{Serum - IgG (g/L)}}{\text{CSF - albumin (mg/L)} \,/\, \text{Serum - albumin (g/L)}}$$

$$\text{CXCL13 ratio} = \frac{\text{CSF - CXCL13 (pg/mL)*1000}}{\text{Serum - CXCL13 (pg/mL)}}$$

$$\text{CXCL13/Albumin ratio} = \frac{\text{CSF - CXCL13 (pg/mL)}}{\text{CSF - albumin (g/L)}}$$

The CXCL13/Albumin ratio was determined similarly to the previously described CSF-CXCL13/total CSF protein quotient in order to compensate for possible differences in CSF protein due to blood-brain-barrier dysfunction (13-14). For the C6 test in CSF, a Lyme index (LI) was calculated by dividing the OD value by an assay cut off value based on a calibrator OD plus 0.3 similar to the manufacturer's instruction for serum samples.

Statistical analyses

In order to determine and compare the ability of different parameters to discriminate between diagnostic groups, cut off values and areas under curve (AUC) were calculated using the groups definite LNB and non-LNB (Table 1) in receiver-operating-characteristic (ROC) analyses using MedCalc, version 11.2.1.0. Alternative cut off values were also determined for CSF-C6 (dilution 1:20) based on mean LI values plus 3 standard deviations (SD) in all non-LNB patients (Table 1, group 4; n=92) and a subset of these 92 patients that also lacked serum anti-borrelia antibodies (n=63). Statistical comparisons of symptom duration between groups were performed using Mann-Whitney's U-test in Statistica, version 8.0. A p-value < 0.05 was considered significant.

Results

Patients and clinical findings

A total of 261 patients were included and divided into four groups (Table 1) based on original results of CSF ABA and the presence of pleocytosis. Clinically these four groups could be interpreted as either definite LNB (group 1), possible LNB (group 2 and 3) or non-LNB (group 4) according to the European Federation of Neurological Societies (EFNS) guidelines (18). Details on sex, age, clinical presentation and duration of symptoms at diagnostic lumbar puncture are presented in Table 1. In group 4, non-LNB, 34 of 92 patients presented with symptoms other than head/neck pain, cranial nerve palsy or radiculitits. In 25 of these 34 patients clinical information in medical charts were available for review. Paresthesias or neuropathies were noted in 11 of these 25 patients. Six patients were investigated for suspected dementia, four for fatigue or vertigo, three for unspecific pain and finally one patient for weakness and possibly a Guillain-Barré syndrome.

Groups 2 and 3, with possible LNB, were then further sub-grouped according to the original findings regarding absence (2a, 3a) or presence (2b, 3b) of anti-borrelia antibodies in serum (Table 2). Based on all available original laboratory results together with information from medical records regarding symptoms and symptom durations the probability of actually suffering from ongoing LNB was re-assessed and estimated in these sub-groups. In group 2a, positive for CSF ABA only, one out of six patients was positive in both IgM and IgG AI and showed a symptom duration of 1.1 weeks, while the remaining five were positive in IgG AI only and had symptom durations of 0.1-4.3 weeks. In group 2b, positive for CSF ABA and with serum anti-borrelia antibodies, 12 of 13 patients were positive in IgG CSF ABA only and the remaining patient was IgM positive only. The median symptom duration in group 2b was 5.4 weeks, significantly longer compared to the other sub-groups (2a, 3a and 3b) using Mann-Whitney's U-test ($p \leq 0.046$, data not shown in table). In group 3a, presenting with only

Table 2. Patient characteristics at diagnostic lumbar puncture in 45 cases of possible Lyme neuroborreliosis (groups 2 and 3 from Table 1).

	Group 2a *n=6*	Group 2b *n=13*	Group 3a *n=10*	Group 3b *n=16*
CSF Anti-borrelia antibodies[a]	+	+	−	−
CSF Pleocytosis	−	−	+	+
Serum Anti-borrelia antibodies	−	+	−	+
Men	5	9	9	10
(%)	(83)	(69)	(90)	(62)
Women	1	4	1	6
(%)	(17)	(31)	(10)	(38)
Median age (years)	37.5	57	35.5	7
range	(18-58)	(40-76)	(5-62)	(2-58)
Head/neck pain	1	4	3[b]	9
(%)	(17)	(31)	(30)	(56)
Cranial nerve palsy	2	1	4[b]	13
(%)	(33)	(7.7)	(40)	(81)
Radiculitits	2	4	1[b]	1
(%)	(33)	(31)	(10)	(6.3)
Other symptom	2	6	2[b]	0
(%)	(33)	(46)	(20)	(0)
Median duration of symptoms before LP (weeks)	2.6	5.4	1.4[b]	1.0
range	(0.1-4.3)	(1.0-77.0)	(0.1-7.9)	(0.3-8.6)

n = Numbers.
CSF = Cerebrospinal fluid.
[a] Either borrelia-specific antibody index or intrathecal anti-borrelia antibodies.
Pleocytosis = Total white blood cell count ≥ $6*10^6$/L CSF.
EFNS = European Federation of Neurological Societies.
LNB = Lyme neuroborreliosis.
[b] Data missing on 1 patient in group 3a.
Patients could have one or more of the symptoms head/neck pain, cranial nerve palsy and radiculitis.
Patients with none of the above symptoms were classified as “other symptom”.

pleocytosis, four of ten patients were children, age 5–8 years, and three of these four children presented between July and October after verified or suspected tick bites with either facial palsy or meningitis for 0.1 to 4.0 weeks. Four other patients in group 3a, age 34–49 years, presented with paresthesias, impaired sensibility, balance disorder or optic neuritis or a combination of these and a suspect diagnosis of multiple sclerosis (MS) had been noted in medical records. One of the two remaining patients in group 3a presented with facial palsy a few days after blisters caused by a probable herpes zoster infection, data was missing on the final patient in group 3a. In group 3b, with pleocytosis and serum anti-borrelia antibodies, 13 of 16 patients presented with cranial nerve palsy (facial nerve) and one additional patient developed facial nerve palsy one day after lumbar puncture. All 14 patients with facial palsy were children at the age of 2–13 years, showing short symptom duration (range 0.3–2.1 weeks) investigated between June and October (data not shown in Table).

Laboratory and statistical findings

Results and distributions for the various laboratory tests are shown in scatter plots per diagnostic group in Figure 1. The diagnostic performance based on ROC analyses of group 1 (definite LNB) and 4 (non-LNB) are shown in Table 3 showing sensitivity, specificity, AUC and best performance cut off values for each parameter. The calculated cut off values of LI and their diagnostic performance for CSF-C6 antibodies are also shown (Table 3). AUC for IgM index was significantly larger than both IgG index and albumin quotient ($p<0.001$ for both comparisons, data not shown in Table). No significant differences in AUC were noted among the various ways of expressing CSF-CXCL13 either by itself or divided by Serum-CXCL13 or CSF-albumin. In 114 of 124 definite LNB cases CSF-CXCL13 concentrations were $\geq$ 500 pg/mL and in eight of the 93 non-LNB cases CSF-CXCL13 concentrations exceeded 10 pg/mL (Figure 1).

Table 3. Diagnostic performance of various laboratory parameters in serum / cerebrospinal fluid in Lyme neuroborreliosis using group 1 (n=124) as definite cases of Lyme neuroborreliosis and group 4 (n=92) as control cases (see Table 1 for definition of groups).

Analysis	Cut off (>)	Sensitivity (%)	Specificity (%)	ROC AUC
Total-IgM Index	0.234	100	92	0.987
Total-IgG Index	0.643	87	75	0.841
CSF-S Albumin ratio (mg/g)	9.7	78	91	0.922
CSF-CXCL13 (pg/ml)	142	98	98	0.984
CSF-S CXCL13 ratio * 1000	354	99	96	0.991
CSF-CXCL13/Albumin (ng/g)	163	98	95	0.966
CSF-C6 LI	0.680	94	98	0.989
CSF-C6 mean LI + 3 SD of 92 negative controls[a]	0.602	94	97	N/A
CSF-C6 mean LI + 3 SD of 63 negative controls[b]	0.092	99	88	N/A
CSF-C6 (undiluted) LI[c]	5.8	87	100	0.966

ROC = Receiver operating characteristic analysis.
AUC = Area under curve.
CSF = Cerebrospinal fluid.
S = Serum.
LI = Lyme index, calculated by dividing sample optical density (OD) value with calibrator OD value plus 0.3.
SD = Standard deviation
[a] Lacking CSFpleocytosis, intrathecal anti-borrelia antibodies or negative anti-borrelia antibody index.
[b] Lacking CSFpleocytosis, intrathecal anti-borrelia antibodies or negative anti-borrelia antibody index, serum anti-borrelia antibodies.
N/A = Not applicable.
[c] 22 samples missing, ROC analysis was performed using 109/124 samples from group 1 and 85/92 samples from group 0.
The Immunetics C6 Lyme ELISA kit was performed on CSF using a sample dilution of 1:20 if not otherwise specified.

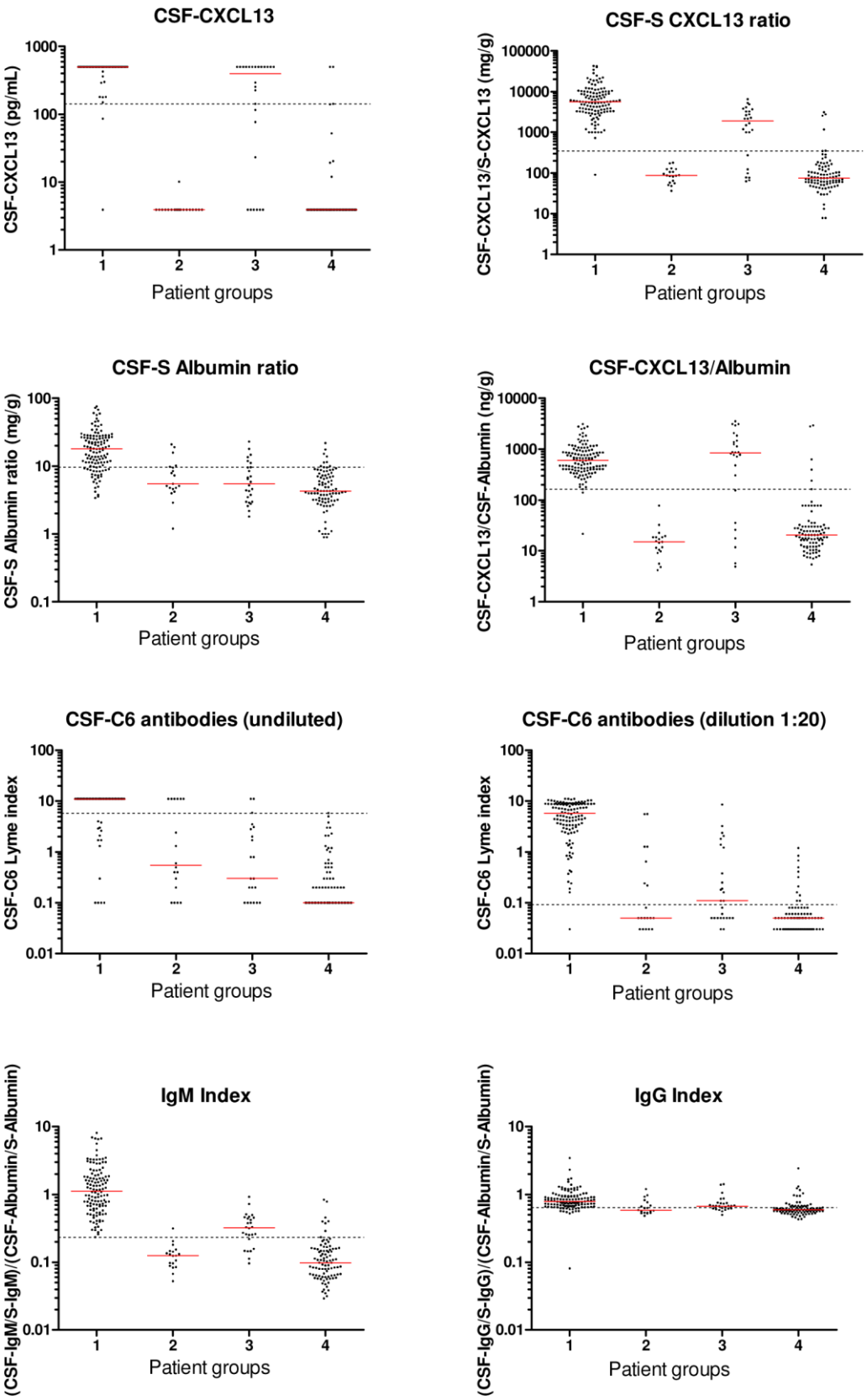

Figure 1: Laboratory results in 261 patients investigated for suspected Lyme neuroborreliosis divided into 4 groups. CSF = Cerebrospinal fluid. S = Serum. LNB = Lyme neuroborreliosis. Group 1 = Definite LNB. Group 2 = Possible LNB. Group 3 = Possible LNB. Group 4 = Non-LNB. See Table 1 for group definitions. Red horizontal bar represents median. Dotted line represents cut off according to Table 3, for the diluted CSF-C6 analysis the cut off at Lyme index = 0.092 is shown.

Regarding the non-LNB cases with elevated CSF-CXCL13 concentrations, only two of eight were found to have CSF-CXCL13 levels of 143 pg/mL or more, i.e. above the cut off. Both these patients were children; one at the age of five presenting with cranial nerve palsy of unknown duration and negative for serum anti-borrelia antibodies, the other was 13 years old with head/neck pain since five days and positive for serum anti-borrelia antibodies. Both these patients were also positive in the total IgM index. Another two non-LNB patients had CSF-CXCL13 of approximately 140 pg/mL; one 47 years old with weakness and myalgia since six weeks and negative in total IgM index, the other at two years of age with head/neck pain since five weeks and positive in total IgM index. Both were positive for serum anti-borrelia antibodies. The remaining four non-LNB patients had CSF-CXCL13 concentrations below 53 pg/mL.

In seven cases (five in group 1 and two in group 3b) both Serum- and CSF-CXCL13 concentrations were ≥ 500 pg/mL and in an additional case in group 1 Serum-CXCL13 was ≥ 500 pg/mL while the corresponding CSF-CXCL13 was 363 pg/mL. In these eight cases CSF-Serum CXCL13 ratios could not be reliably interpreted as either positive or negative according to cut off. However, in all eight cases the CSF-CXCL13/Albumin ratios were ≥ 704 ng/g (range 704–2593) and total-IgM index were ≥ 0.300 (range 0.300–1.48), *i.e.* above the cut off levels for both markers according to the ROC analyses implying that the these cases should be judged as positive. As an extra precaution, ROC analyses were also performed for CSF-CXCL13, CSF-Serum CXCL13 ratio and CSF-CXCL13/Albumin ratio after excluding these eight cases, and these analyses showed the same results (data not shown in Table). For further calculations positive results were assumed in these eight cases also for the CSF-Serum CXCL13 ratio resulting in a sensitivity of 99% and a specificity of 96% for the CSF-Serum CXCL13 ratio based on group 1 and 4, and three of the four children in group 3a and all 14

children in group 3b would be positive. In four patients with suspected MS in group 3a, the CSF-Serum CXCL13 ratios varied between 5.8 and 66.8 mg/g, *i.e.* all were negative.

The use of CXCL13 levels in serum led to a poor separation of the groups with a sensitivity of 47%, a specificity of 80% and an AUC of 0.634 (data not shown in Table). Regarding C6-antibodies, a tendency was noted towards a higher AUC for the diluted CSF C6 test compared to the undiluted C6 test (p=0.064, data not shown in Table), although 22 sample results of undiluted CSF-C6 antibodies were not available for ROC analysis. Various calculations were tried in order to obtain a C6 antibody index (19). The LI values were used in combination with concentrations of albumin, total-IgM and total-IgG in both CSF and serum, but were not included due to poor performance in ROC analyses perhaps because of the partly non-linear relation between LI (OD) values and concentrations of specific antibodies in ELISA tests. As it has previously been shown that the OD values alone in CSF in the C6 test can be used successfully, we analysed the LI values without further corrections (15). Finally, applying the various obtained cut off values, the positivity rates for the studied parameters were calculated in the various diagnostic groups (Table 4 and 5). When a combination of CSF-S CXCL13 ratio and CSF-C6 (dilution 1:20) antibodies (with cut off at LI 0.092) was applied and considered positive only when both markers were positive, a sensitivity of 99% in group 1 (definite LNB) and a specificity of 98% in group 4 (non-LNB) was reached. Using the same definition, one of the four children in group 3a and 12 of the 14 (86%) children, in group 3b, would be positive (data not shown in Table). No significant difference in AUC was noted comparing ROC analyses of CSF-Serum CXCL13 ratio alone to the combination of CSF-Serum CXCL13 ratio and CSF-C6 antibodies (dilution 1:20) in groups 1 and 4.

Table 4. Proportion positive (%) per parameter and diagnostic group in 261 patients investigated for suspected Lyme neuroborreliosis based on cut off values obtained in Table 3.

	Group 1 *n=124*	Group 2 *n=19*	Group 3 *n=26*	Group 4 *n=92*
CSF Anti-borrelia antibodies[a]	+	+	−	−
CSF Pleocytosis	+	−	+	−
Clinical interpretation according to EFNS guidelines	Definite LNB	Possible LNB		Non-LNB
Total-IgM Index	100	5.3	73	7.6
Total-IgG Index	87	37	65	25
CSF-S Albumin ratio	78	26	23	8.7
CSF-CXCL13	98	0.0	65	2.2
CSF-S CXCL13 ratio	99	0.0	73	4.3
CSF-CXCL13/Albumin	98	0.0	73	5.4
CSF-C6 cut off at LI 0.680	94	21	27	2.2
CSF-C6 cut off at LI 0.092	99	37	54	12
Both CSF-CXCL13/Albumin and CSF-C6 cut off at LI 0.092	98	0.0	54	3.3
Both CSF-S CXCL13 ratio and CSF-C6 cut off at LI 0.092	99	0.0	54	2.2
	n=109	*n=18*	*n=21*	*n=85*
CSF-C6 (undiluted)	87	33	14	0.0

n = Numbers.
CSF = Cerebrospinal fluid.
[a] Either borrelia-specific antibody index or intrathecal anti-borrelia antibodies.
Pleocytosis = Total white blood cell count ≥ $6*10^6$/L CSF.
EFNS = European Federation of Neurological Societies.
LNB = Lyme neuroborreliosis.
S = Serum.
LI = Lyme index, calculated by dividing sample optical density (OD) value with calibrator OD value plus 0.3.
The Immunetics C6 Lyme ELISA kit was performed on CSF using a sample dilution of 1:20 if not otherwise specified.

Table 5. Proportion positive (%) per parameter and diagnostic group in subgroups of patients with signs of possible Lyme neuroborreliosis (n= 45, see Table 2 for details regarding subgroups) based on cut off values obtained in Table 3.

	Group 2a *n=6*	Group 2b *n=13*	Group 3a *n=10*	Group 3b *n=16*
CSF Anti-borrelia antibodies[a]	+	+	−	−
CSF Pleocytosis	−	−	+	+
Borrelia-specific antibodies in serum	−	+	−	+
Total-IgM Index	0.0	7.7	60	81
Total-IgG Index	0.0	31	70	62
CSF-S Albumin ratio	33	23	20	25
CSF-CXCL13	0.0	0.0	10	100
CSF-S CXCL13 ratio	0.0	0.0	30	100
CSF-CXCL13/Albumin	0.0	0.0	30	100
CSF-C6 cut off at LI 0.680	0.0	31	0.0	44
CSF-C6 cut off at LI 0.092	0.0	54	10	81
Both CSF-CXCL13/Albumin and CSF-C6 cut off at LI 0.092	0.0	0.0	10	81
Both CSF-S CXCL13 ratio and CSF-C6 cut off at LI 0.092	0.0	0.0	10	81
	n=6	*n=12*	*n=8*	*n=13*
CSF-C6 (undiluted)	33	33	0.0	23

n = Numbers.
CSF = Cerebrospinal fluid.
[a] Either borrelia-specific antibody index or intrathecal anti-borrelia antibodies.
Pleocytosis = Total white blood cell count ≥ $6*10^6$/L CSF.
EFNS = European Federation of Neurological Societies.
LNB = Lyme neuroborreliosis.
S = Serum.
LI = Lyme index, calculated by dividing sample optical density (OD) value with calibrator OD value plus 0.3.
The Immunetics C6 Lyme ELISA kit was performed on CSF using a sample dilution of 1:20 if not otherwise specified.

Discussion

The results from this study of 261 patients in the south-east of Sweden confirm that CXCL13 in CSF is a reliable marker for LNB. CXCL13, expressed as CSF-Serum CXCL13 ratio, reached a sensitivity of 99% and a specificity of 96% when studying group 1, definite LNB, and group 4, non-LNB, in line with previously published results (12-13, 20). Although negative in CSF ABA, 14 children of 16 patients in group 3b were interpreted as early LNB due to short symptom duration, typical symptoms, the presence of pleocytosis and serum anti-borrelia antibodies, a classification which is also supported by the fact that a similarly defined group of children with possible LNB showed borrelia-specific T-cell responses to the same extent as children with definite LNB (21). Notably, all 14 children had a positive CSF-Serum CXCL13 ratio. For C6 we found that results from diluted CSF tended to show a higher AUC than the results from undiluted CSF, suggesting the use of diluted CSF, although this notion needs to be further tested. However, based on this finding, and also due to a complete set of results for C6 antibodies from diluted CSF, the results from the diluted CSF were preferred in further calculations. Thus, by using mean LI values plus 3 SD of a well defined control group, the diagnostic performance of CSF-C6 antibodies in this material, with a sensitivity of 99% and a specificity of 88%, was similar to that found by Skarpaas et al. (15), but differed form the lower sensitivity found by Vermeersch et al. (16), perhaps due to differences in selection criteria.

Levels of CXCL13 in LNB have previously primarily been reported as a ratio between CSF-CXCL13 and total protein in CSF to compensate for damage to the blood-brain-barrier (12-14). However, the CSF-Serum CXCL13 ratio has been reported to differentiate patients with neurosyphilis (NS) even better from LNB patients compared with the CSF-CXCL13/total protein ratio. The CSF-Serum CXCL13 ratios are higher in LNB compared to NS patients primarily due to higher serum CXCL13 levels in NS (22-23). Although the two diseases can

mimic each other clinically (24), LNB is generally much more common than NS in areas where LB is prevalent. For instance, the incidence of LB has been reported to be 69 per 100.000 inhabitants and year in the south of Sweden (1), compared with an incidence of 1.9 for syphilis, according to the Swedish Institute for Infectious Disease Control. However, a diagnostic method should not depend on mere differences in incidence rates. Furthermore, increased levels of CSF-CXCL13 have also been reported in MS, albeit at lower levels, in general, compared with LNB (12-13, 25-27). Although CXCL13 in CSF is a more specific marker for LNB compared to pleocytosis, which may be found in many other central nervous system diseases such as bacterial and viral meningitis and various inflammatory central nervous system diseases (13), the increased CXCL13 levels found in MS and NS point to the benefit of combining results of CXCL13 with intrathecally produced anti-borrelia antibodies in order to gain diagnostic specificity. Thus, we evaluated the combination of CSF-Serum CXCL13 ratio in parallel with CSF-C6 antibodies. Using the combination of CSF-Serum CXCL13 and CSF-C6 antibodies a specificity of 98% was reached although not statistically different from the CSF-Serum CXCL13 ratio alone with a specificity of 96% in group 4 in this material.

According to the reassessment of patients with possible LNB (groups 2 and 3), ongoing LNB was probable in 1 of 6 patients in group 2a, although this individual was negative for CSF-CXCL13, CXCL13 ratios and CSF-C6 antibodies. Based on the lack of pleocytosis and longer symptom duration, patients in group 2b were considered to have had a previous episode of LNB or possibly prolonged symptoms from LNB, which is supported by the fact that CSF ABA may persist for months and years even after clinical recovery of LNB (8). Indeed, CSF-C6 antibodies were also found, but only in 7 of 13 patients (54%, with cut off at LI 0.092), whereas CSF-CXCL13 and CXCL13 ratios were negative in all patients most likely reflecting the lack of inflammatory activity in these patients, in line with the absence of

CSF pleocytosis. In group 3a, three of four children with CSF pleocytosis possibly had LNB and all three were positive in CSF-Serum CXCL13 ratio and one of them also positive for CSF-C6 antibodies. In group 3b, as mentioned, 14 children were considered likely to have LNB, and all were positive for CSF-CXCL13.

Interestingly, total-IgM index showed a high diagnostic performance, as previously described (28) and may add diagnostic information in cases where results of CSF-Serum CXCL13 ratio and CSF-C6 antibodies are inconclusive.

This study is limited by its retrospective design, but this is compensated to a great extent by the large size of the study material in comparison with the previous studies of these markers in the diagnosis of LNB (12-16, 22-23). The specificity of the assays may have been overestimated because only patients suspected of having LNB on clinical grounds were included in the evaluation. Results could also be affected by the prolonged storage in –20° C before analyses, and for albumin, total concentrations of IgM, IgG and C6 antibodies samples had been refrozen and thawed before analysis. However, all samples were handled in the same way and previous studies indicate that serum proteins such as albumin and immunoglobulins are fairly robust and insensitive to storage in –20° C during time intervals such as in this study as well as to repeated freeze-thaw cycles (29-32). According to our limited investigation we found the precision to be less than that reported by the manufacturer regarding CXCL13. However, as a minimum of overlap between definite and non-LNB cases was found in this study, we regard this level of precision to be acceptable. In order to obtain as many observations as possible for CXCL13 and the combination with C6 antibodies, we decided not to exclude the eight cases showing CXCL13 levels above the measuring range (and due to lack of sample the final concentration could not be settled). The CSF-Serum CXCL13 ratios were assumed positive based on the positive results in these cases for CSF-CXCL13/Albumin ratios and total-IgM index as well as the fact that the same ROC results

were obtained comparing groups 1 and 4 with or without these eight patients. For future use however, final concentrations, possibly requiring dilutions of CXCL13 in serum and CSF, should be obtained in order to reliably interpret the CSF-Serum CXCL13 ratio.

In conclusion, this large study confirms CXCL13 in CSF as a reliable marker of LNB. The CSF-Serum CXCL13 ratio differentiates LNB well from other conditions and also detects highly probable cases of LNB among children with short symptom duration where CSF ABA are still negative. To improve the diagnostic routine in cases of suspected LNB, we recommend the use of CSF-CXCL13.

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