Chapter 5

Discriminating Lyme neuroborreliosis from other neuro-inflammatory diseases using levels of CXCL13 in cerebrospinal fluid

Nathalie D. van Burgel[1]
Floor Bakels[2]
Aloys C.M. Kroes
Alje P. van Dam[3]

J Clin Microbiol. 2011 May;49(5):2027-30
Published as research note

[1] Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands
[2] Department of Neurology, Leiden University Medical Center, Leiden, the Netherlands
[3] Department of Medical Microbiology, Onze Lieve Vrouwe Gasthuis, Amsterdam, the Netherlands

Abstract

Lyme neuroborreliosis is a severe but treatable disease. Intrathecal production of chemoattractant CXCL13 has been suggested to be a good biomarker for diagnosing LNB. Our aim was to determine levels of the CXCL13 biomarker in cerebrospinal fluid (CSF) in LNB and several groups of patients with inflammatory neurological diseases, in order to evaluate performance of a CXCL13 ELISA for diagnosing LNB.

Fifty-eight adult and pediatric LNB patients, 36 Lyme non-neuroborreliosis cases, 93 infectious meningitis/ encephalitis controls and 74 neurological controls were tested for levels of CXCL13 in CSF.
Levels of CXCL13 were highly elevated in the patients who presented with LNB. Sensitivity using an optimal cut-off of 250pg/ml CSF was 88%. Children (n=24) had lower levels of CXCL13 intrathecally than the adult population (n=35), this difference was not significant (median=932 compared to median 1678; p=0.4).
In the controls elevated levels of CXCL13 in CSF were seen in several groups of patients. Overall specificity was 89%; this was lowest in the HIV positive population where it was 77%.
After treatment there was a rapid decline in CXCL13 levels in CSF of LNB patients. Determining CSF CXCL13 as a marker for follow up after adequate treatment seemed promising.

Determining levels of CXCL13 as a marker for LNB can be useful, but should be interpreted with care especially in the immunocompromised patient and in the patient with an autoimmune disorder. HIV infection should be excluded in individuals with elevated levels of CXCL13 in CSF.

Introduction

Lyme neuroborreliosis (LNB) is the neurologic manifestation of an infection with the tick-borne spirochete *B. burgdorferi* sensu lato. LNB can clinically present with many symptoms varying from classical presentations like facial nerve paralysis and Bannwarth's syndrome to a range of neurological disorders [111]. Diagnosing Lyme neuroborreliosis is a challenge for the clinician. Calculating the *B. burgdorferi* specific antibody index (AI) is the preferable method, though the sensitivity can be variable, ranging from 66-79% [354, 356, 562].

Measuring intrathecal levels of CXCL13 has been suggested as a potential biomarker for diagnosing LNB. CXCL13 is produced by antigen presenting cells and is a selective chemoattractant for B-cells and B-helper T-cells. High levels of CXCL13 have been detected in muscle tissue in chronically *B. burgdorferi* infected rhesus macaques [563]. In vitro dendritic cells were able to produce high amounts of CXCL13 when they were exposed to *B. burgdorferi* antigens [564]. In vivo production of CXCL13 could be confirmed in non-human primates infected with *B. burgdorferi* intrathecally, where ectopic germinal centers were formed in the brain when high levels of CXCL13 were detected [564]. CXCL13 has been found to be expressed at high levels in pooled cerebrospinal fluid (CSF) from human LNB patients (219ng/g total protein) by a cytokine array, while in pooled CSF from subjects with non-inflammatory neurological disease levels were barely detectable (<1.7ng/g protein) [380]. In a patient cohort of acute LNB patients CXCL 13 levels were highly elevated in all 37 definite LNB cases and no or minimal elevation was seen in the non LNB controls (n=8) [381]. In another study 28 LNB cases had significantly elevated levels of CXCL13 compared to neurological and infectious controls. Some infectious controls had high levels of CXCL13 intrathecally, but overall sensitivity and specificity for LNB using a cut off of 337ng/g total protein were 96% and 97% respectively [382]. Case reports describing early diagnosis of LNB using CXCL13 levels in CSF have already been reported [565, 566]

Our aim was to determine the diagnostic potential of levels of intrathecal CXCL13 to distinguish acute and late LNB from other central nervous system diseases in the pediatric and adult population.

Materials and methods

Clinical samples

Subjects from June 2004 to May 2010 were identified retrospectively using the laboratory information management system from the Leiden University

Medical Center (Leiden), OLVG Hospital (Amsterdam), IZORE Center for Infectious Diseases (Leeuwarden), Academic Medical Center (Amsterdam) and the Isala clinic (Zwolle).
Cerebrospinal fluid (CSF) samples from 58 LNB patients before treatment were included. Criteria for diagnosing LNB patients were; no other cause of meningitis and three of the following four characteristics: positive serology at presentation, pleocytosis, evidence of intrathecally produced specific *B. burgdorferi* antibodies with IDEIA™ Lyme Neuroborreliosis (OXOID, Cambridge, UK) and objective neurological complaints with favorable outcome after treatment according to the EUCALB guideline [415]. Definite LNB were patients from this group who had a pleocytosis and a positive antibody index. Probable LNB were patients who either had pleocytosis or a positive antibody index [414].
Ninety-three CSF samples from subjects with an infectious cause of meningitis/ encephalitis which consisted of cases with; neurosyphilis (n=12), tuberculosis (TBC) meningitis (n=1), pneumococcal meningitis (n=2), Listeria meningitis (n=1), cryptococcal meningitis (n=8), toxoplasma encephalitis (n=14), intrathecal aspergilloma (n=4), human immunodeficiency virus (HIV) meningitis (n=6), varicella zoster virus (VZV) Bell's Palsy and encephalitis (n=11), herpes simplex virus-1 (HSV-1) encephalitis (n=6), enterovirus meningitis (n=23), parechovirus meningitis (n=3) and tick borne encephalitis (TBE) (n=2). Sixty-two CSF samples were collected from neurological inflammatory diseases including; multiple sclerosis (MS) (n=27), polyneuritis (n=16), idiopathic facial nerve paralysis (FNP) (n=18), acute disseminated encephalomyelitis (ADEM) (n=1). Thirty-six CSF samples were collected from Lyme borreliosis subjects that did not have LNB defined as absence of pleocytosis and lack of objective neurological complaints. Twelve CSF samples were collected from subjects with non inflammatory neurological complaints consisting of trauma patients, dizziness and headache without evident diagnosis. Additionally seven CSF samples from HIV patients that had no neurological complaints or evidence of an intrathecal infection were collected. Demographic data was collected for all patient groups; age at diagnosis, sex, CSF findings at diagnosis (intrathecal leukocyte count, percentage lymphocytes, erythrocytes, glucose and total protein levels). For LNB patients the clinical presentation, duration of symptoms at presentation and report of an erythema migrans (EM) were documented.

CXCL13 ELISA

CSF samples were tested in the Quantikine Human CXCL13/ BLC/ BCA-1 Immunoassay (R&D systems, Minneapolis, USA) according to manufacturer's protocols. Briefly, 50 µl of CSF was diluted with 100µl of Assay Diluent RD1S, transferred to a monoclonal anti-CXCL13 precoated microtiterplate, and

incubated for 2 hours at room temperature (RT). Each well was aspirated and washed four times with 400µl wash buffer. Two hundred microliters of a horseradishperoxidase conjugated mouse monoclonal anti-human CXCL13 was added to each well and incubated for 2 hours at RT. Each well was aspirated and washed four times with 400µl wash buffer. Two hundred microliters of 3,3',5,5'-tetramethylbenzidine was added to each well for 30 minutes at RT in the dark, then 50µl of 1M sulphuric acid was added to each well and absorption was read at 450nm within 30 minutes. CXCL13 concentration was calculated with a standard curve, included in each set of samples assayed. Samples that were outside the linear range of measurement and standard curve were diluted with Assay Diluent and retested accordingly. Analysis was performed with samples in pg CXCL13/ml CSF.

Antibody index

Sera and CSF samples were tested in the IDEIA™ Lyme Neuroborreliosis kit according to manufacturer's protocol (OXOID, Cambridgeshire, UK). Briefly, serum samples were diluted 1:200, CSF samples were diluted 1:4 and both tested in a total volume of 100 µl in the ELISA. Samples were incubated for 60 minutes and subsequently rinsed four times with 350 µl of wash buffer and 100 µl of flagellum conjugate was incubated for one hour. Wells were aspirated and rinsed four times with 350 µl of wash buffer and 100 µl of substrate was added and incubated for 10 minutes after which 100 µl stop solution is added and absorbance is measured within 30 minutes at 450nm. Antibody index (AI) is calculated as (OD_{csf}/OD_{serum}) * $(OD_{csf}-OD_{serum})$. The CSF is positive for IgG or IgM if the OD_{csf} IgG or IgM is > 0.150. The AI is positive when the CSF is positive and the AI_{IgG} or $AI_{IgM} \geq 0.3$.

Statistical analysis

Statistical analysis was performed using SPSS (version 17.0). The Pearson bivariate correlation algorithm was used to calculate correlation. The Mann-Whitney U test was used to compare levels of CXCL13 between groups. P values <0.05 were considered significant. A receiver operating characteristic (ROC) curve was used to calculate discriminatory capacity of CXCL13 levels. The maximal value of the Youden index was applied to choose an optimal cut-off (J_{max}= (sensitivity + specificity)-1).

Results

Clinical findings

Patient characteristics are depicted in table 1. Of the 58 LNB patients 91% of the LNB patients presented within 6 months of the start of complaints; the range was 7 days to 48 months. Forty-one percent of LNB patients were children. Most LNB patients presented with a facial nerve paralysis (60%) or meningoradiculitis (26%). Seventeen percent of LNB patients reported experiencing an EM before or at presentation. In patients with LNB 98% had pleocytosis and 79% was positive for intrathecal production of borrelia specific IgG and / or IgM. Overall 45 patients could be classified as definite LNB and 13 as probable LNB.

	n	Male/Female (%)	Mean age yrs (SD)	Mean leukocyte count /µl CSF (SD)
Lyme neuroborreliosis	58	65/35	38 (25)	138 (159)
Lyme borreliosis	36	47/53	51 (17)	1 (1)
Infectious meningitis/ encephalitis controls	93			
T. pallidum	12	83/17	40 (8)	40 (80)
C. neoformans	8	37/63	55 (14)	81 (69)
Aspergillosis	4	25/75	41 (8)	2 (3)
Toxoplasmosis	14	43/57	35 (20)	1 (0)
Bacterial meningitis				
S. pneumoniae	2	50/50	41 (6)	3032 (893)
L. monocytogenes	1	0/100	61	1280
M. tuberculosis	1	0/100	4	25
Viral meningitis/encephalitis				
HIV	6	50/50	43 (8)	51 (45)
VZV	11	45/55	51 (23)	130 (174)
HSV1	6	33/67	55 (30)	46 (51)
Enterovirus	23	61/39	13 (17)	217 (381)
Parechovirus	3	0/100	0	1 (1)
TBE	2	50/50	37 (4)	59 (12)
Neurologic controls	74			
Facial nerve paralysis	18	56/44	48 (19)	42 (150)
Multiple sclerosis	27	33/67	35 (13)	14 (17)
Polyneuritis	16	56/44	45 (17)	17 (22)
ADEM	1	0/100	21	266
Neurologic non-inflammatory controls	12	25/75	50 (15)	4 (7)
HIV controls	7	86/14	50 (8)	1 (1)

Table 1: Demographic data and CSF leukocyte count per patient group

CXCL13 levels in CSF at diagnostic lumbar puncture

CXCL-13 ELISA on all samples was performed as indicated. CSF CXCL13 levels are depicted in figure 1. Median levels of CXCL13 were significantly elevated in LNB patients compared to the Lyme non-neuroborreliosis controls (median 1183 and median 3pg/ml; p<0.001). CSF levels of CXCL13 were lower in children than in adults with LNB, but this difference was not significant (median 932 pg/ml compared to median 1678 pg/ml; p=0.4).

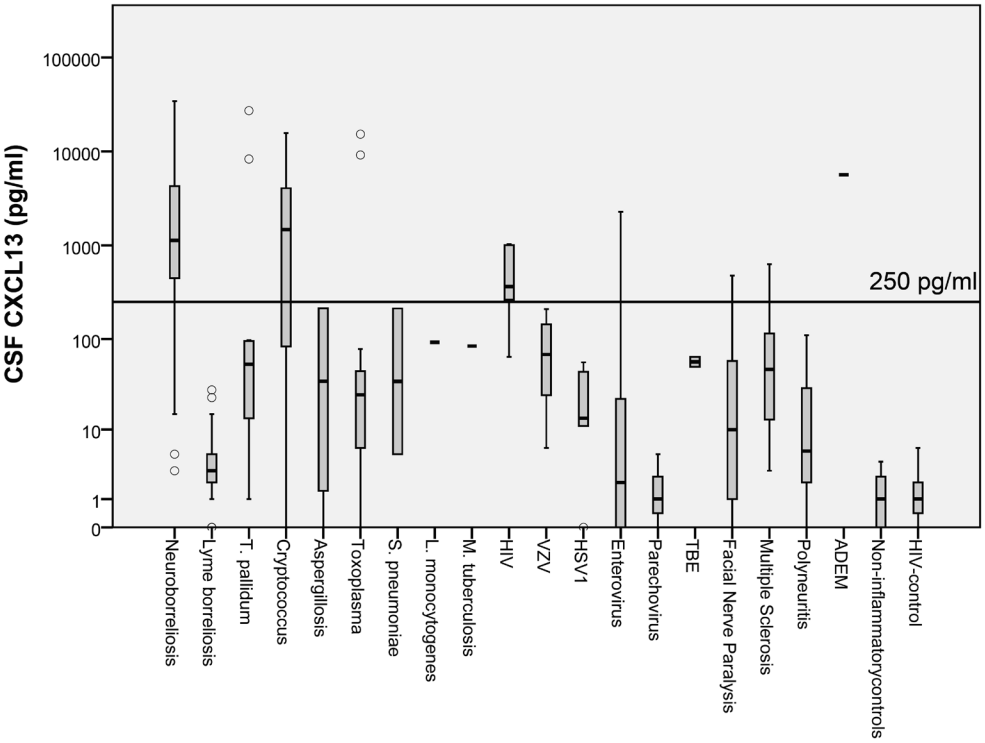

Figure 1: Levels of CXCL13 in CSF of LNB patients and controls. Horizontal lines indicate medians, bars represent interquartile ranges, lines represent 95% confidence interval and bullets represent outliers. Reference line is located at cut-off 250pg/ml as was determined by ROC curve analysis for optimal sensitivity and specificity for discriminating LNB patients from controls.

In figure 2 it is shown that in LNB patients CSF levels of CXCL13 correlated with the amount of leukocytes in the CSF at presentation (R^2=0.172; p<0.01). No correlation was found between duration of complaints to levels of CXCL13 at presentation in early and late LNB (R^2=0.11; p=0.4). This was also true when only the adult population with early LNB were analyzed (R^2=0.13; p=0.6). The

duration of storage of a sample before testing at -20°C did not result in lower levels of CXCL13 in the LNB group (R^2=0.015; p=0.4).

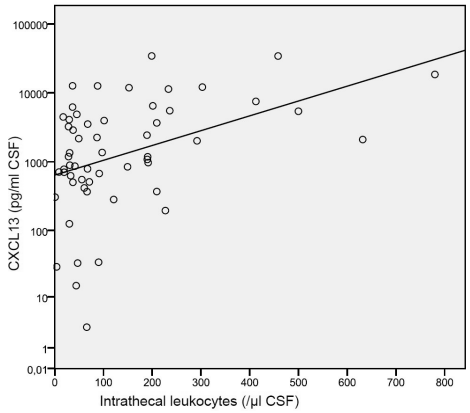

Figure 2: Correlation of intrathecal leukocytes with the level of CXCL13 measured intrathecally in LNB patients. Reference line is a regression line, the correlation is significant (R2=0.172, p=0.001)

Sensitivity of CXCL13 for diagnosing LNB

ROC analysis revealed an optimal cut-off of 250pg/ml which resulted in 88% sensitivity and 89% specificity (figure 3). Results for positive and negative levels of CXCL13 using the cut-off of 250pg/ml in LNB patients and controls are shown in table 2. Seven LNB patients had CXCL13 levels under 250pg/ml CSF. This group consisted of 3 children en 4 adults, of them 5 had early LNB and 2 had late LNB. Lowering the cut-off to 30pg/ml would result in a sensitivity of 97%, but in a reduction in specificity to 65% in the overall population. In the neurologic non-inflammatory controls and the LB controls none of the subjects had levels of CXCL13 over 30pg/ml.

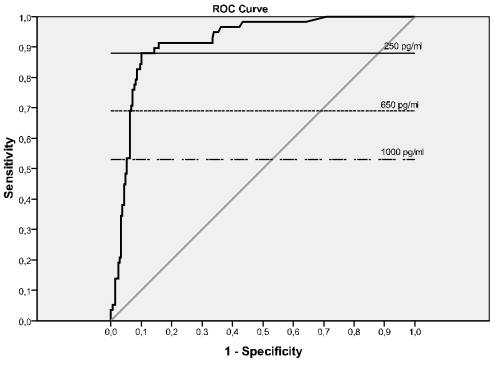

Figure 3: ROC curve analysis using levels of CXCL13 to discriminate between LNB and all controls. Horizontal lines show three different cut-off values for levels of CXCL13. Optimal cut-off was located at 250pg/ml.

Specificity of CXCL13 in the controls

Eleven percent of the controls had CXCL13 levels over 250pg/ml CSF (table 2). None of the CXCL13 positive controls had positive serology for Lyme. All positive controls are specified in table 3. A remarkable result is the elevated levels of CXCL13 in CSF for some HIV positive patients. As is shown in table 3 the specificity of the assay is higher in the HIV negative controls; overall specificity in the HIV negative population is 92%(153/166) while the specificity in the HIV positive controls is only 77% (34/44). The control group of HIV patients without signs of intrathecal infection did not show elevated CXCL13 levels. As is shown in figure 3 elevation of the cut-off to two randomly chosen cut-offs led to rapid decline of sensitivity.

		LNB (n=58) CXCL13 ≥250pg/ml (%)	**<250pg/ml (%)**	**Controls (n=210) CXCL13 ≥250pg/ml (%)**	**<250pg/ml (%)**
AI	positive	42 (72)	4 (7)	0 (0)	4 (2)
AI	negative	9 (16)	3 (5)	23 (11)	183 (87)
Pleocytosis		50 (86)	7 (12)	19 (9)	76 (36)
No pleocytosis		1 (2)	0 (0)	4 (2)	111 (53)
Definite LNB		41 (71)	4 (7)		
Probable LNB		10 (17)	3 (5)		
No LNB				23 (11)*	187 (89)

Table 2: CXCL13 positivity in patients and controls using a cut-off of 250pg/ml. *No controls with elevated levels of CXCL13 had serum serology positive for Lyme.

Discussion and conclusions

We confirm high levels of intrathecal CXCL13 expression in adult and pediatric patients with LNB; however in contrary to previous publications we also identify other groups of subjects with clinically similar presentation with high levels of CXCL13 in CSF.

Identification of LNB is difficult due to the clinical presentation which has much similarity to other diseases. The current definite diagnosis of LNB consists of a positive AI and elevated CSF leukocytes [414]. However, many patients with LNB present with absence of one of those parameters. This can vary from a very early presentation with negative AI to late presentation with few leukocytes intrathecally [111, 112, 356].

This study is the first to include children in the analysis for CXCL13 levels as a diagnostic marker in LNB. Furthermore it is the first study that studied a large adult and pediatric population of neuro-inflammatory patients as controls.

In this study high levels of CXCL13 were found in LNB patients in 88% of LNB patients. This is a lower sensitivity than what was found in previous studies (96-100%) [380-382]. One possible explanation could be that storing samples for a prolonged time would decrease levels of CXCL13, however in this study no effect on median levels of CXCL13 was found for up to five years of storage.
Another explanation for the lower sensitivity found here could be the different study populations. As is shown in table 2, sensitivity is 91% (41/45) in definite LNB cases where correct diagnosis is imminent due to presence of pleocytosis in combination with a positive AI. In probable LNB cases, where either pleocytosis or a positive AI is absent, the sensitivity was 77% (10/13). Previously these probable cases were not well investigated though the role of CXCL13 as an additional marker in these probable cases specifically could be very important.
Previous studies expressed CXCL13 levels in ng CXCL13 /g total protein in CSF [382]. ROC curve analysis for amount of CXCL13 per milliliter compared to amount CXCL13 per gram of total protein showed a similar AUC in this population (0.91 to 0.90 respectively). Applying the previously proposed cut-off of 337ng/g for identifying LNB patients in this study led to a slightly lower sensitivity and slightly lower specificity of 82% and 88% respectively. One study defined a cutoff of 142 pg/ml of CSF. In our study, such a cutoff led to a sensitivity of 90% and a specificity of 84% [372].

	≥250 pg/ml/ total n	**≥250pg/ml/ total n (HIV+)**	**≥250pg/ml/ total n (HIV-)**
T. pallidum	2/12	2/8	0/4
C. neoformans	6/8	2/4	4/4
Toxoplasma encephalitis	2/14	0/12	2/2*
HIV meningitis	5/6	5/6	-
Enterovirus meningitis	1/23	0/1	1/22**
Facial nerve paralysis	1/18	1/5	0/13
Multiple Sclerosis	5/27	0/0	5/27
ADEM	1/1	0/0	1/1

Table 3: High CXCL13 levels in controls (pg CXCL13/ml CSF). * both subjects were congenital toxoplasmosis patients; **positive is in a subject suffering from hypogammaglobulinemia.

A high prevalence of elevated levels of CXCL13 levels in a number of groups of patients with diseases other than LNB was found in this study. Diseases with

elevated expression of CXCL13 intrathecally have been described previously; patients with MS showed marginally elevated CXCL13 levels [380, 385], bacterial and viral causes of meningitis also resulted in moderate elevation of intrathecal CXCL13 [382]. CXCL13 levels were comparable to what was found in this report. This study reports an elevated expression of CXCL13 intrathecally in several other infections. One of the most remarkable observations is that in HIV positive patients levels of CXCL13 are elevated in several central nervous system infections. Elevated expression of CXCL13 in serum of HIV patients has been reported previously, as well as the combination of altered expression of the receptor and elevated production of the CXCL13 cytokine in vitro [384, 567]. Dysfunction of B cells in HIV patients may lead to high levels of CXCL13, independent of the cause of the meningitis or encephalitis.
Neonates with congenital toxoplasmosis and subjects with cryptococcal meningitis showed very high levels of CXCL13, both most likely due to the prolonged infection and inability to clear the infection. Clinically a cryptococcal infection could resemble a neuroborreliosis, but cryptococcal meningitis will often be found only in immunocompromised individuals. One patient that suffered from hypogammaglobulinemia and chronic enterovirus meningitis had CSF CXCL13 levels of 2278pg/ml. Inability of B-cells to respond adequately to the presented antigen will likely lead to continuous production of CXCL13 and high levels intrathecally in hypogammaglobulinemia patients. In the neurological controls the slightly elevated level of CXCL13 was confirmed in MS subjects. Furthermore the patient with an ADEM had very high levels of CXCL13, most likely due to high dysfunctional activity of B-cells intrathecally. One other patient is of interest; a child with coxsackie B3 enterovirus meningitis with subsequent presentation with Henoch-Schonlein purpura (HSP) displayed above average CXCL13 levels of 249pg/ml. HSP and systemic lupus erythematosis (SLE) are autoimmune disorders that both display B-cell dysfunction. Elevated levels of CXCL13 in serum of SLE patients have been described previously [387]. These results suggest that in subjects with autoimmune disorders involving B-cell dysfunction intrathecal CXCL13 levels can be elevated in the absence of LNB.

Treatment of LNB leads to vast reduction in CXCL13 CSF levels, which makes it a potential marker for studying disease activity and effective clearance after treatment [381, 382]. This application seemed very promising. Further studies including follow-up of patients with objective and subjective symptoms of LNB should be performed.

Determining levels of CXCL13 as a marker for LNB can be useful, but should be interpreted with care because it is not a specific marker, especially in the

immunocompromised patient and in the patient with an autoimmune disorder. HIV infection should be excluded in individuals with elevated levels of CXCL13 in CSF. Determining CSF CXCL13 as a marker for disease activity seems promising, but further research for this application is necessary.

Acknowledgements
We would like to thank Wim Ang, Lesla Bruijnesteijn-van Coppenraat, Sunita Paltansing, Jos Kerremans and Afke Brandenburg for collecting and donating their LNB samples.