The B-lymphocyte chemokine CXCL13 in the cerebrospinal fluid of children with Lyme neuroborreliosis; associations with clinical and laboratory variables

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Abstract:

Background: The B-lymphocyte chemokine CXCL13 is increasingly considered a useful early phase diagnostic marker of Lyme neuroborreliosis (LNB). However, the large variation in level of CXCL13 in the cerebrospinal fluid (CSF) observed in LNB patients is still unexplained. We aimed to identify factors associated with the level of CXCL13 in children with LNB, possibly improving the interpretation of CXCL13 as a diagnostic marker of LNB.

Methods: Children with confirmed and probable LNB were included in a prospective study on CXCL13 in CSF as a diagnostic marker of LNB. The variables age, sex, facial nerve palsy, generalized inflammation symptoms (fever, headache, neck-stiffness and/or fatigue), duration of symptoms, *Borrelia* antibodies in CSF, *Borrelia* antibody index, CSF white blood cells (WBC), CSF protein, and detection of the genospecies *Borrelia garinii* by PCR were included in simple and multivariable regression analyses to study the associations with the CXCL13 level.

Results: We included 53 children with confirmed and 17 children with probable LNB. CXCL13 levels in CSF were positively associated with WBC, protein and *Borrelia* antibodies in CSF in both simple and multivariable analyses. We did not find any associations between CXCL13 and age, sex, clinical symptoms, duration of symptoms, antibody index or the detection of *Borrelia garinii*.

Conclusions: High levels of CSF CXCL13 are present in the early phase of LNB and correlate with the level of CSF WBC and protein. Our results indicate that CSF CXCL13 in children evaluated for LNB can be interpreted independently of clinical features or duration of symptoms.

Keywords: Lyme neuroborreliosis, CXCL13, children, clinical variables, laboratory variables, diagnosis

Introduction:

The tick borne infection Lyme neuroborreliosis (LNB) is caused by spirochetes from the *Borrelia burgdorferi* sensu lato (*Borrelia*) complex entering the central nervous system (CNS) and inducing inflammation when recognized by the host immune system (1, 2). The CNS inflammation in LNB patients is characterized by increased concentration of both pro-inflammatory and regulatory cytokines (1, 3-5), and a marked mononuclear pleocytosis in the cerebrospinal fluid (CSF) dominated by B-lymphocytes and plasma-cells (1, 6-8). The B cell chemokine CXCL13 plays an important role in trafficking of B-lymphocytes to the site of infection (2), and substantially increased concentrations of this chemokine has been measured in the CSF during LNB infections in both adults and children (9-16). CXCL13 in CSF is increasingly considered a useful additional diagnostic marker of LNB, especially in the early phase when the intrathecal production of the *Borrelia* specific antibodies may not yet be detectable (17). However, the wide range in CXCL13 levels observed in adults and children with LNB is still unexplained. Furthermore, a large variability in clinical symptoms, duration of symptoms and intrathecal inflammation characteristics have been described in LNB patients (18-21) and different *Borrelia* genospecies can cause LNB (22-28). It is not known if any of these factors influence the release of CXCL13 in the CSF during LNB. To understand possible mechanisms associated with the CXCL13 release in the CSF and to improve the interpretation of CXCL13 as a diagnostic marker for LNB, identifying factors associated with levels of CXCL13 in LNB is important.

The aim of this study was to explore how clinical and laboratory characteristics are associated with the level of CXCL13 in the CSF of children with LNB.

Material and Methods:

Subjects, data collection and diagnostic classification:

In a prospective multicenter study, all children with symptoms suggestive of LNB aged three months to 18 years who were admitted to the pediatric departments of five hospitals in south west Norway from autumn 2011 to spring 2014 were invited to participate. Children who had been given antibiotics prior to admission were excluded. At admission, children or parents were interviewed with a standardized questionnaire, and standard serum and CSF samples were taken. Children were classified into different diagnostic groups with high or low likelihood of having LNB prior to the analyses of CXCL13, as described previously (9). In the present study we included only children classified as either confirmed LNB (CSF pleocytosis and intrathecally produced antibodies against *Borrelia*, expressed as a positive antibody index, or probable LNB (CSF pleocytosis, negative antibody index and either positive *Borrelia* antibodies in serum or a recent history of erythema migrans). Both groups were included as LNB patients in the further analyses.

Laboratory analyses:

The CSF analyses of white blood cells (WBC), protein and *Borrelia* antibodies were performed at each local laboratory, as previously described (9). One ml CSF from each child were stored frozen on -70 ^{o}C for later analyses of CXCL13 and *Borrelia* genospecies determination, both analyses performed at the Hospital of Southern Norway Trust, Kristiansand, Norway. The CXCL13 analyses were performed by an enzyme-linked immunosorbent assay (Quantakine, R&D Systems, Minneapolis, MN, USA), previously described in more detail (9). *Borrelia* genotyping was performed by five single-plex real-time polymerase chain reaction

(PCR) assays. As previously reported, *Borrelia garinii* (*B. garinii*) was the predominant genospecies associated with LNB in these children (22). In most CSF samples the concentration of *Borrelia* spirochetes were low, and in some samples possibly under the detection limit of the PCR assays used. Consequently, a negative PCR result for *B. garinii* did not guaranty the absence of *B. garinii*.

Variables:

Variables possibly associated with the CXCL13 level in CSF were classified in two groups. (A) Demographic and clinical variables: age, sex, presence of facial nerve palsy, symptoms of generalized inflammation (fever, headache, neck-stiffness or fatigue) and duration of symptoms, and (B) laboratory/CSF variables: CSF WBC, CSF protein, *Borrelia* antibody index (AI), CSF *Borrelia* IgG antibodies, CSF *Borrelia* IgM antibodies and detection of *B. garinii* in the CSF.

Statistical analyses:

We performed simple and multivariable linear regression analyses for associations between CXCL13 in the CSF and the variables (A and B). The continuous variables CXCL13, duration of symptoms and CSF WBC were all severely skewed. We therefore used the natural logarithm of these variables in the analyses. From the regression models we report effect estimates with 95% confidence intervals (CI), p-values from Wald tests of no effects, R^2 for each model, and change in R^2, i.e. ΔR^2, by inclusion of each variables in the models. For ease of interpretation some of the effect estimates are presented as percent difference in medians (29).

The variable "detection of *B. garinii*" was only included in the simple regression analyses. Biologically and theoretically, the *Borrelia* bacteria induce CXCL13 release in the CSF

which results in the following: Increased level of CSF WBC (recruitment of B-lymphocytes into the CSF), and in turn elevated levels of CSF protein (due to production of immunoglobulins by plasma cells, matured from the recruited B-lymphocytes) (2, 7). Thus, the level of CXCL13 will influence the level of WBC and protein in the CSF (variables B). In multivariable models of possible associations between CXCL13 and other variables, adjusting for CSF WBC and protein may cause collider bias. Consequently, multivariable analyses were performed with adjustment for the variables A and not B.

Statistical analyses were performed using SPSS Statistics 23 (IBM, New York, USA). A p-value < 0.05 was considered significant.

Results:

In total, 77 children with LNB were eligible for inclusion. Seven children were excluded as their CSF study sample had been temporarily stored on -20 C^{o} for weeks before further storing on -70 C^{o}. The remaining 70 children (53 with confirmed LNB and 17 with probable LNB) were included in the present study and their clinical and laboratory characteristics are presented in Table 1.

In the simple linear regression analyses, the level of CXCL13 in the CSF was associated with the level of WBC and protein in the CSF and the detection of *Borrelia* IgG and IgM antibodies in the CSF (Table 2). CXCL13 was not significantly associated with age, sex, facial nerve palsy, generalized inflammation symptoms, duration of symptoms, antibody index or detection of *B. garinii* (Table 2). CXCL13 remained positively associated with CSF WBC, CSF protein, *Borrelia* IgG and *Borrelia* IgM after adjusting for age, sex, facial nerve palsy and

duration of symptoms, of which the associations were strongest with CSF WBC and CSF protein, as judged by ΔR^2 (Table 2). We also performed additional adjustment for generalized inflammation symptoms, including 60 children in the multivariable regression model, but the results were unchanged. Log CSF WBC correlated with CSF protein; Pearson correlation coefficient 0.587 ($p = 0.001$). The relations between CXCL13 and CSF WBC, CSF protein, age and duration of symptoms are shown graphically in Figure 1. In the adjusted models children with *Borrelia* IgG in the CSF had 300% (95% CI 38-1060%) higher median CXCL13 values than those without *Borrelia* IgG, whereas children with *Borrelia* IgM in the CSF had 212% (34-640%) higher median CXCL13 levels than those without *Borrelia* IgM.

Discussion:

In this study in children with LNB, the levels of CXCL13 in the CSF were positively associated with the levels of WBC and protein and the detection of *Borrelia* IgG and IgM antibodies in the CSF. We did not find associations between CXCL13 and age, sex, type of symptoms, duration of symptoms, antibody index or detection of *B. garinii* in the CSF.

Associations between CXCL13 and WBC, protein and *Borrelia* antibodies in the CSF

Several prior studies have found a similar positive association between CXCL13 and WBC in the CSF in both adults and children with LNB, as we did (30-33). Our study cannot determine cause-effect relationships between CXCL13 and CSF WBC, but growing evidence suggests that CXCL13 is the driver of CSF pleocytosis in LNB. An experimental model by Rupprecht et al. have shown that CXCL13 is the main regulator of B-cells in CSF in LNB (7).

Moreover, cases of possible early phase LNB with increased CXCL13 levels prior to the pleocytosis have also been reported (9, 34).

One of many possible mechanisms behind the correlation between CXCL13 and protein in the CSF, is recruitment of B-lymphocytes developing into plasma cells and eventually producing *Borrelia* antibodies (immunoglobulins) (2). The positive association between CXCL13 and intrathecal *Borrelia* IgG and IgM (Table 2) in our study, supports this hypothesis. According to studies by Reiber, proteins in the CSF originate from blood (80%) and CNS (20%) (35, 36). Moreover, changes in the CSF protein concentration may be due to alterations in serum protein levels, intrathecal release of immunoglobulins, blood/CSF barrier properties or changes in the CSF flow-rate and drainage (36, 37). Possibly, other factors than immunoglobulins contributes to the correlation between CXCL13 and CSF protein in LNB, but this cannot be determined by our study.

Taken together, CXCL13, WBC and protein seems to be strongly correlated and possibly all reflect the state of inflammation in LNB patients. Whether these correlations have any clinical implications is unclear, but Markowicz et.al. have suggested that applying a linearized cut-off for CXCL13 dependent on the CSF WBC level could be a novel approach in the diagnosis of LNB (31).

Associations between CXCL13 and clinical features of LNB in children

The strong association between CSF CXCL13 and WBC and protein cannot explain the large variety in the level of CXCL13 observed in LNB patients. Children with LNB present with variable symptoms and signs, often categorized in groups with either facial nerve palsy, symptoms of generalized inflammation / mild meningism, or both of these symptoms (18, 20).

Symptoms may also vary according to age and sex (38). However, as for previous studies, we could not identify clinical variables such as symptoms, age or sex predicting the level of CXCL13 in children with LNB (14, 30).

In adults with LNB it seems that high levels of CXCL13 correlate with short duration of symptoms (13), whereas this correlation has not been confirmed in children (14, 30). In general, children with suspected LNB are investigated after shorter duration of symptoms compared to adults (19, 20) and this may explain why there was no correlation between the level of CXCL13 and duration of symptoms in our study. Nevertheless, children in our study had substantially elevated levels of CXCL13 in the CSF already after a few days of symptoms (Figure 1), suggesting an early and pronounced release of CXCL13 in the CNS during LNB. Interpretation of diagnostic markers often depend on the duration of the disease. It is therefore important to understand how the diagnostic marker is induced by both the disease of interest and possibly by other relevant diseases. A few experimental studies have shown that the CXCL13 release in LNB is caused by binding of *Borrelia* spirochetes to Toll-like receptor 2 (TLR2) on local immune cells (monocytes, macrophages and dendritic cells) (39, 40). Other pathogens, such as *Streptococcus pneumonia*, can also bind to TLR2 (41), but the CXCL13 release is much less pronounced (40). This is supported by the findings of Pilz et al., who reported elevated levels of CXCL13 in the CSF of patients with both bacterial (including *S. pneumonia*) and viral neuroinfections (42), but with a less pronounced and a more gradual increase than previously reported in LNB patients (10). We have previously shown that children with non-Lyme aseptic meningitis have substantially lower levels of CXCL13 compared to children with LNB with similar duration of symptoms (9). Experimental studies on Rhesus Macaques have shown that the CXCL13

concentration peaks between one and three weeks after intrathecal inoculation with *Borrelia* (43). Thus, compared to other CNS infections, the CXCL13 release in LNB is early and pronounced.

As far as we are aware, the relation between the CXCL13 level in the CSF and different *Borrelia* genospecies causing LNB, has not been studied before. We did not find an association between the CXCL13 level and detection of the *B. garinii* genospecies in the CSF of children with LNB, all though absence of the *B. garinii* genospecies was an uncertain variable in our study.

CXCL13 is released in the CSF in the early phase of LNB, before the *Borrelia* antibody index (11, 17) and sometimes even before pleocytosis can be detected in the CSF (9, 34). The major clinical application of CXCL13 may therefore be the discrimination between LNB and non-Lyme aseptic meningitis when the antibody index is still negative (9, 17). This scenario is not uncommon in children with suspected LNB, who are often investigated early and share clinical features with those of non-Lyme aseptic meningitis. These patients are often characterized as probable or possible LNB (17, 19, 20). We have previously shown that 18/18 children with probable LNB would have been diagnosed as LNB if CXCL13 with a low cut-off level was applied for the diagnosis (9). We can still not explain the large variety in the levels of CXCL13 in children with LNB. However, our results indicate that CXCL13 is associated with LNB per se and not with specific clinical features of the disease or the causing genospecies, making this a good candidate for a diagnostic marker. For pediatricians considering the LNB diagnosis, this may implicate that CXCL13 levels in the CSF can be interpreted independently of clinical features or duration of symptoms at the time of lumbar puncture.

Strengths and limitations

The strength of this study is the prospective inclusion of patients, the predefined diagnostic criteria and that all CSF samples included in this study were treated equally. Even though this is one of the largest studies on LNB in children, the confidence intervals for some of the effect estimates are wide and we cannot rule out that some of the non-significant associations may be clinically relevant. Another limitation was that six CSF samples contained insufficient amount of CSF for determining AI, even though *Borrelia* antibodies were present in the CSF. Therefore, we chose to present both *Borrelia* antibodies in the CSF and the AI as variables in the regression analyses. One could speculate whether CXCL13 would have been positively associated with AI if AI analyses could have been performed in these six samples.

Conclusion:

This study has shown that the CXCL13 release in CSF during LNB infection in children is pronounced and present in the early phase of the disease. High levels of CXCL13 are associated with high levels of WBC and protein and detection of *Borrelia* antibodies in the CSF. We could not identify any demographic or clinical variables associated with the level of CXCL13 in children with LNB. Thus, CXCL13 is associated with LNB, but probably not with specific features of the disease or duration of symptoms, supporting the role as a good diagnostic marker for LNB.

Notes:

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Conflict of interest:

The authors declare that they have no competing interests.

Ethical approval:

The study was approved by the Regional Committee for Medical Health and Research Ethics in Western Norway.

Informed consent:

For each child, one of the parents provided written informed consent to participate.

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Table 1 Demographic and clinical characteristics (A) and laboratory characteristics in the CSF (B) of 70 children with LNB

Variables		
A	Age years, mean (SD) [range]	7.1 (2.75) [2 - 14]
	Sex, male/female (% male)	38/32 (54)
	Facial nerve palsy, yes/no (% yes)	45/25 (64)
	Generalized inflammation symptoms [a], y/n (% y)	55/5 (92) [n=60, b]
	Duration of symptoms days, median (IQR) [range]	7 (3, 19) [1 - 120]
B	CSF CXCL13 pg/ml, median (IQR) [range]	2641 (944, 8434) [7 - 63212]
	CSF WBC 10^6/L, median (IQR) [range]	164 (57, 282) [10 - 733]
	CSF protein g/L, median (IQR) [range]	0.51 (0.36, 0.81) [0.16 - 1.61]
	CSF *Borrelia* AI (IgG and/or IgM), y/n (% y)	53/11 (82) [n=64, c]
	CSF *Borrelia* IgG y/n (% y)	57/13 (81)
	CSF *Borrelia* IgM y/n (% y)	44/26 (63)
	Detection of *B. garinii* in CSF, y/n (% y)	26/43 (38) [n=69, d]

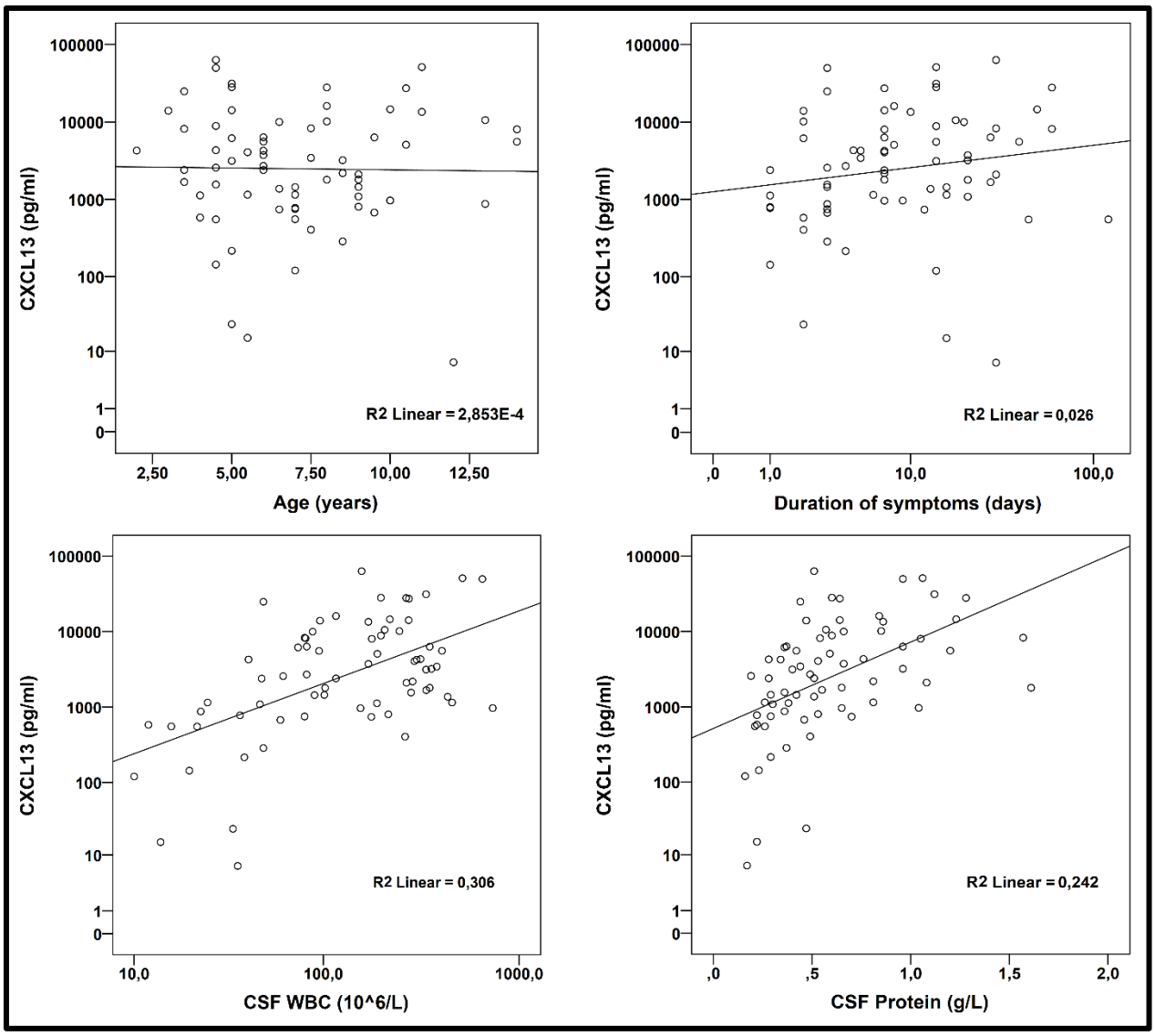
Abbreviations: CSF: cerebrospinal fluid, LNB: Lyme neuroborreliosis, SD: standard deviation, IQR: interquartile range, WBC: white blood cell count, AI: antibody index, IgG: Immunoglobulin G, IgM: immunoglobulin M.

[a] Headache, fever, neck stiffness or fatigue. [b] Missing information to confirm absence of symptom in ten children, thus n=60. [c] Insufficient amount of CSF in sample for successful AI test in six children, thus n=64. [d] Insufficient amount of CSF in sample for analyses of genospecies determination in one child, thus n=69.

Table 2 Simple and multivariable linear regression analyses of associations between different demographic and clinical (A) and laboratory/CSF variables (B) and the log transformed level of CXCL13 in the CSF of 70 children with LNB

		Simple regression analyses			Multivariable regression analyses adjusting for: age, sex, FNP and log duration of symptoms		
	N	Beta (95% CI)	P-value	R^2 (%)	Beta (95% CI)	P-value	ΔR^2
A. Demographic and clinical variables							
Age	70	-0.01 (-0.17, 0.15)	0.88	0.0	-0.03 (-0,2, 0.14)	0.72	0.2
Sex = boy	70	0.10 (-0.78, 0.97)	0.83	0.1	0.09 (-0.82, 0.99)	0.85	0.0
Facial nerve palsy (y)	70	-0.33 (-1.24, 0.57)	0.47	0.4	0.12 (-1.07, 1.32)	0.84	0.0
Generalized inflam-mation symptoms (y)	60	0.76 (-1.02, 2.53)	0.40	1.2	0.51 (-1.46, 2.48)	0.61	1.1
Log duration of Symptoms, days	70	0.28 (-0.11, 0.66)	0.15	3.0	0.32 (-0.19, 0.84)	0.21	2.3
B. CSF variables							
Log WBC 10^6/L	70	0.96 (0.61, 1.31)	**<0.001**	30.7	1.03 (0.67, 1.38)	**<0.001**	32.9
Protein g/L	70	2.65 (1.51, 3.79)	**<0.001**	24.1	3.37 (2.06, 4.67)	**<0.001**	28.4
Borrelia AI (y)	64	1.03 (-0.17, 2.23)	0.092	4.5	1.02 (-0.29, 2.32)	0.124	4.3
Borrelia IgG (y)	70	1.39 (0.32, 2.45)	**0.012**	9.0	1.46 (0.28, 2.63)	**0.016**	8.5
Borrelia IgM (y)	70	1.14 (0.29, 2.0)	**0.010**	9.4	1.10 (0.21, 1.98)	**0.016**	8.5
Detection of *B. Garinii* (y)	69	0.03 (-0.86, 0.92)	0.94	0.0	0.18 (-0.75, 1.12)	0.70	-0.4

Abbreviations: CSF: cerebrospinal fluid, LNB: Lyme neuroborreliosis, y: yes, WBC: white blood cell count, AI: antibody index, *B. garinii*: *Borrelia garinii*, R^2: R square.



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403 **Figure 1** Relation between the level of CSF CXCL13 and age, duration of symptoms, CSF WBC and CSF protein

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