Diagnostic value of cerebrospinal fluid chemokine ligand 13 (CXCL13) levels for viral and autoimmune encephalitis

Keywords
CXCL13, Lyme neuroborreliosis, autoimmune encephalitis, viral encephalitis

Abstract

Introduction
Chemokine ligand 13 (CXCL13) has been reported to be a valuable diagnostic biomarker in Lyme neuroborreliosis (LNB). However, its utility in the diagnostics of viral and autoimmune (AE) encephalitis still remains unclear.

Material and methods
We have measured CXCL13 concentrations in cerebrospinal fluid (CSF) samples collected from 21 patients with viral encephalitis (17 cases of herpes simplex viral (HSV) and four of enteroviral (EV) encephalitis), six patients with AE (five subjects with antibodies anti-NMDAR and one with anti-GABA) and compared them to those found in patients with LNB (seven subjects), multiple sclerosis (eight cases) and ten controls comprised of subjects without neuroinflammation.

Results
Patients with neuroinflammation had a mean level of CXCL13-CSF of 105 pg/ml compared to a value of 29 pg/ml for controls. The highest mean level of CXCL13 in CSF was detected in LNB patients (233 pg/ml) whereas the lowest was in controls (29pg/ml). A significant upregulation of CXCL13-CSF levels in LNB patients was determined when compared to viral encephalitis, MS and controls. A positive correlation between the increased levels of chemokine and cell count in CSF was found in all patients ($r=0.6496$; $p<0.0001$), as well as, in LNB group when tested alone ($r=0.8428$; $p=0.0173$). Positive correlation with CSF protein levels was confirmed in all patients ($r=0.7216$; $p<0.0001$), and separately in LNB ($r=0.8573$; $p=0.0137$) and AE patients ($r=0.8885$; $p=0.0180$).

Conclusions
The utility of CXCL13 measurements in CSF for LNB diagnosis was confirmed. No specific patterns in CXCL13-CSF levels were associated with viral and autoimmune encephalitis.

Diagnostic value of cerebrospinal fluid chemokine ligand 13 (CXCL13) levels for viral and autoimmune encephalitis

Running title: CXCL13 in viral and autoimmune encephalitis

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ABSTRACT

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Materials and methods: We have measured CXCL13 concentrations in cerebrospinal fluid (CSF) samples collected from 21 patients with viral encephalitis (17 cases of herpes simplex viral (HSV) and four of enteroviral (EV) encephalitis), six patients with AE (five subjects with antibodies anti-NMDAR and one with anti-GABA) and compared them to those found in patients with LNB (seven subjects), multiple sclerosis (eight cases) and ten controls comprised of subjects without neuroinflammation.

Results: Patients with neuroinflammation had a mean level of CXCL13-CSF of 105 pg/ml compared to a value of 29 pg/ml for controls. The highest mean level of CXCL13 in CSF was detected in LNB patients (233 pg/ml) whereas the lowest was in controls (29pg/ml). A significant upregulation of CXCL13-CSF levels in LNB patients was determined when compared to viral encephalitis, MS and controls. A positive correlation between the increased levels of chemokine and cell count in CSF was found in all patients ($r=0.6496$; $p<0.0001$), as well as, in LNB group when tested alone ($r=0.8428$; $p=0.0173$). Positive correlation with CSF protein levels was confirmed in all patients ($r=0.7216$; $p<0.0001$), and separately in LNB ($r=0.8573$; $p=0.0137$) and AE patients ($r=0.8885$; $p=0.0180$).

Conclusions: The utility of CXCL13 measurements in CSF for LNB diagnosis was confirmed. No specific patterns in CXCL13-CSF levels were associated with viral and autoimmune encephalitis.

KEYWORDS: CXCL13, viral encephalitis, autoimmune encephalitis, Lyme neuroborreliosis

INTRODUCTION

Despite recent improvements in clinical management of encephalitis, the syndrome still remains challenging due to its possible severe course and mortality [1]. In more than 50% of cases the etiologic factor of neuroinflammation remains undetermined due to the vast number of infectious and noninfectious causative agents resulting in often time-consuming and complicated diagnostic procedures [2-5]. Moreover, different pathogens may generate similar clinical pictures like Lyme neuroborreliosis (LNB) and enteroviral (EV) encephalitis [6]. The leading causes of CNS inflammation are viral infections (45-69% of all cases), mostly triggered by human herpesvirus type 1 (HSV) and EVs, which constitute up to 24% and 6-25% of confirmed encephalitis cases, respectively [5, 7, 8]. Up to 20% of encephalitis cases are immune-mediated with the presence of anti-neuronal or anti-glial antibodies, in particular autoantibodies against N-methyl-d-aspartate receptor (anti-NMDAR); [9].

Due to the variety of encephalitis types it is vital to develop new tests and biomarkers which will allow for early diagnosis and their differentiation. Chemokine (C-X-C motif) ligand 13 (CXCL13) seems to be a new and promising biomarker, as its utility already has been confirmed in LNB diagnostics [2, 10, 11]. High chemokine concentrations in cerebrospinal fluid (CSF) were found to be strongly associated with early phase of acute LNB and correlated with changes of typical CSF's neuroinflammation markers (e.g. pleocytosis and CSF protein levels); [12]. Changes in CXCL13-CSF concentrations were also suggested to serve as a prognostic biomarker for multiple sclerosis (MS) progression [13] while, CXCL13 serum levels were found to be associated with treatment response in MS [14, 15]. Among other immune diseases, CXCL13 was proposed as a possible marker and future therapeutic target [16] in systemic lupus erythematosus [17], rheumatoid arthritis [18], Sjögren's syndrome

[19], myasthenia gravis [20] and inflammatory bowel disease (IBV); [21]. CXCL13-CSF levels as high as in LNB were also reported in neurosyphilis, and therefore it was proposed to use it as a diagnostic marker useful in patients also infected with Human immunodeficiency virus (HIV) as the virus itself might trigger pleocytosis [22-24].

In the present study we have analyzed CXCL13-CSF levels in viral and autoimmune encephalitis (AE), as well as, in LNB and MS patients, to establish its possible diagnostic role in these types of encephalitis.

MATERIALS AND METHODS

We have analyzed 52 adult (≥18 yrs.) patients who were admitted to two Warsaw hospitals: i) patients with encephalitis and LNB were hospitalized in the Hospital for Infectious Diseases in Warsaw; ii) MS patients and control subjects were hospitalized in the Department of Neurology, Medical University of Warsaw. Written informed consent was obtained from all study participants or from their relatives if the given subject was unable to give the consent himself. The study was approved by the Internal Review Board of the Medical University of Warsaw.

ENCEPHALITIS

Viral encephalitis patients consisted of subjects from a previously described surveillance study focused on etiology of encephalitis [8]. Encephalitis was defined by the presence of altered mental status, decreased level of consciousness, seizures or focal neurological signs together with at least one abnormality in the cerebrospinal fluid (CSF): white blood cell count ≥ 4 cells/mm^2 and/or protein level ≥ 40 mg/dl [8]. Only patients with confirmed encephalitis caused by HSV and EV were included in the study. Viral etiology was

determined based on positive viral detection performed in CSF using non-commercial PCRs [25, 26].

LYME NEUROBORRELIOSIS

Patients with LNB were a part of a previously described research in which CXCL13-CSF levels were measured [6] however, for the purpose of normalization, these measurements were performed once again in the same run with other samples analyzed in the present study.

LNB was diagnosed according to the guidelines recommended by the European Federation of Neurological Societies (EFNS); [27]. Besides clinical criteria, *B. burgdorferi s.l.* specific antibodies were detected in paired sera and CSF samples with the use of *Borrelia* IgM/IgG ELISA tests (Biomedica, Vienna, Austria). Positive ELISA detections were confirmed by Western Blot (recomLine *Borrelia* IgM and recomLine *Borrelia* IgG; Mikrogen Diagnostik, Neuried, Germany).

PCR detecting *Borrelia* flagellin gene (flaB) was employed as described previously [28]. In short, DNA was extracted from 200µl of serum and CSF samples with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and then subjected for amplification using GeneProof *Borrelia* PCR Kit IVD (GeneProof, Brno, Czech Republic) with the following PCR conditions: initial denaturation at 95°C for 5 min. followed by 35 cycles of denaturation at 95°C for 30 sec. annealing at 52°C for 30 sec. elongation at 72°C for 80 sec. and a final elongation at 72°C for 7 min. Second round of PCR included 35 cycles of denaturation at 95°C for 20 sec. followed by annealing at 55°C for 20 sec. and elongation at 72°C for 60 sec. Amplification was visualized on 1.5% agarose gel and if positive, products were commercially sequenced and analysed using MEGA ver.11 [29].

Among seven patients with LNB included in the study, three were classified as confirmed and four as possible LNB based on the combination of clinical and diagnostics results described previously [6].

AUTOIMMUNE ENCEPHALITIS

Study group included previously described subjects tested for the presence of AE autoantibodies in CSF: NMDAR, contactin-associated protein 2 (CASPR2), glutamate receptors (type AMPA1/2), leucine-rich glioma-inactivated protein 1 (LGI1), dipeptidyl aminopeptidase-like protein 6 (DPPX) and GABA B receptor [30]. Antibodies were identified using Autoimmune Encephalitis Mosaic 6 panel (Euroimmune, Germany) supported by the Nikon Eclipse 80i (Nikon, Japan) microscope, employed to read fluorescence under the magnifications of ×20 and ×40.

Autoimmune-triggered encephalitis was confirmed in six patients. In five (83.33%) of them antibodies against NMDAR were detected in CSF, whereas one had anti-GABA.

MULTIPLE SCLEROSIS AND CONTROL PATIENTS

Eighty-three patients admitted to the hospital with initial diagnosis of MS after further examination were divided into two groups: patients with confirmed MS and control group defined as subjects without CNS inflammation. MS patients were diagnosed according to the revised McDonald criteria introduced in 2017 [31] with lumbar puncture being performed as part of their routine diagnostics.

Eight patients were diagnosed with MS and 10 were classified as controls in the study. Among control patients one remained undiagnosed while, four cases had peripheral neuropathy, three had peripheral vertigo and two patients had retinopathy.

CXCL13 MEASUREMENTS

CXCL13 in CSF was measured according to the instructions provided by the manufacturer using CXCL13 ELISA (Euroimmun, Lübeck, Germany). Optical density (OD) was analyzed on Multiskan FC (Thermo Fisher Scientific, Waltham, MA, USA) photometer. Based on manufacturer's recommendations the following levels of CXCL13-CSF corresponded to a specific interpretation: <20 pg/ml - normal values, values from 20 to 29 pg/ml - borderline range, values 30-100 pg/ml - elevated, and >100 pg/ml – LNB if corresponding clinical symptoms are present. Statistical comparisons between groups were made using ANOVA test, while the possible correlation was determined using Pearson's test.

RESULTS

Overall, 52 patients were included in the study, consisting of 18 women and 34 men with mean age of 39 years. Subjects can be divided into two major groups: patients with neuroinflammation (42 patients) and controls - 10 subjects with absence of neuroinflammation, as well as, neuroinfection and demyelination.

Among 42 patients with neuroinflammation twenty-one patients were classified as viral encephalitis; in 17 (81%) and in four (19%) the causative agent of inflammation was determined to be HSV and EV, respectively. Viral loads of HSV in CSF ranged from 60 to 341 copies per milliliter and were higher than those found for EV (12-60 copies/ml); (Table 1). In one patient with confirmed LNB, EV was detected with viral load of 220 copies/ml.

Among all analyzed subgroups LNB patients had the highest mean values of cytosis (116 cells/µl) and protein (102 mg/dl) in CSF, as well as, the percentage of detected lymphocytes (93%). Based on routine CSF analysis the mean cytosis was 7 and 2 cells/µl in MS group and controls, respectively.

Patients with neuroinflammation had a mean level of CXCL13-CSF of 105 pg/ml compared to a value of 29 pg/ml for controls (Table 1). The highest mean level of CXCL13 in CSF was detected in LNB patients (233 pg/ml), followed by AE (153 pg/ml), viral encephalitis cases (72 pg/ml), patients with MS (46 pg/ml) and controls (29pg/ml).

The threshold criteria of >100 pg/ml for the chemokine in CSF was met for four LNB and viral encephalitis patients each, as well as in three AE cases. A significant ($p<0.05$) upregulation of CXCL13-CSF levels in LNB patients was determined when compared to viral encephalitis, MS and controls (Figure 1).

A positive correlation between the increased levels of chemokine and cell count in CSF was found in all patients ($r=0.6496$; $p<0.0001$), as well as, in LNB group when tested alone ($r=0.8428$; $p=0.0173$). Similar results were obtained when CXCL13 levels were analyzed together with CSF protein levels. Positive correlation was confirmed in all patients ($r=0.7216$; $p<0.0001$), and separately in LNB ($r=0.8573$; $p=0.0137$) and AE patients ($r=0.8885$; $p=0.0180$); (Table 1).

DISCUSSION

CXCL13, previously known as B cell attracting chemokine 1 (BCA-1); [32] is constitutively expressed by dendritic cells (DC), monocytes, macrophages and stromal cells in secondary lymphoid organs such as spleen, lymph nodes and Peyer's patches [33, 34]. The chemokine acts via the cognate CXCR5 receptor (Burkitts lymphoma receptor; CD185), which is expressed on mature B lymphocytes, DC and T follicular helper cells (Tfh); [35]. CXCL13 plays a primary role in the development and organization of lymphoid tissues as it regulates migrations of B cells and subsets of T cells to lymphoid follicles [36]. Furthermore,

CXCL13/CXCR5 axis seems to play a role in the formation of ectopic lymphoid tissues within CNS, and therefore it is involved in the development of neuroinflammation [10].

In the present study we analyzed CXCL13-CSF level in patients with viral and autoimmune encephalitis, as well as, in those with LNB and MS in whom they were previously reported to be elevated, to verify its diagnostic utility for these types of neuroinflammation.

The highest chemokine levels in CSF have been detected in LNB patients but widely ranged from 23 to 657 pg/ml. Although elevated, CXCL13-CSF concentrations found in our study were lower than those described for untreated acute LNB (mean value of 15,149 pg/ml) [37] or presented by Erhart *et al.* where median CXCL13 level of was 3,920 pg/ml [38]. The most likely explanation would be that CXCL13 levels are high when tested immediately after sample collection and they are decreasing along with the CSF storage time [22, 37].

Although higher CXCL13-CSF mean concentrations were found in 42 patients with neuroinflammation compared to controls no significant differences were reported. At the same time significant changes in CXCL13-CSF levels were observed between LNB patients and separately controls, MS and viral encephalitis patients. Our data is consistent with the results published by other research groups [37, 39-41], and confirms the utility of CXCL13-CSF testing as being a valuable biomarker of LNB. Our results also support the claim that CXCL13-CSF can be helpful to differentiate LNB from other neuroinflammation, at the same time showing that its upregulation cannot be ruled out in case of single encephalitis patients [38]. Furthermore, we have shown that the CSF chemokine levels are above recommended thresholds in 19% and 50% viral and autoimmune encephalitis cases, respectively.

Viral encephalitis subjects included in the study encompassed patients with confirmed HSV and EV neuroinfection. Only few previous studies analyzed CXCL13-CSF in viral encephalitis cases apart, rather subjecting into the analysis viral and bacterial cases combined together as “infectious encephalitis” [42].

Current data on CXCL13 activity in CNS in viral encephalitis is inconsistent. In our study CXCL13-CSF levels did not significantly differ from controls. In turn, Smíšková *et al.* showed that median CSF levels of the chemokine are significantly higher in patients with viral encephalitis including HSV, EV and tick-borne encephalitis (TBE) cases when compared to controls [43]. In another study significantly higher CXCL13-CSF levels were also reported in patients with encephalitis caused by HSV and varicella zoster virus (VZV) than in controls consisting of subjects with non-inflammatory neurological diseases [38]. In contrast, viral encephalitis patients infected by human herpesvirus 6, VZV, EV and HSV were found to have similar CXCL13-CSF levels as controls [44].

A high proportion (50%) of AE patients had CXCL13-CSF upregulations, however chemokine concentrations did not significantly differ from those in controls, that could be explained by a small AE sample size used in the study. In one of the largest retrospective study (conducted on 167 patients with anti-NMDAR encephalitis) an increased ($p<.001$) CXCL13 levels in CSF were reported when compared to 25 patients with non-inflammatory disorders [45]. Significant upregulations were also described by Kothur *et al*. in pediatric patients with anti-NMDAR encephalitis, acute disseminated encephalomyelitis (ADEM) or EV encephalitis in regards to 20 non-inflammatory neurological controls mostly consisting of cases with cerebral palsy monogenic movement disorders [46]. It was found that CXCL13 levels in CSF are the highest at early stage of anti-NMDAR encephalitis and they gradually

decreases over time which could explain the outcome of our investigation while some of our patients where chronic AE cases [47].

Although specific diagnostic patterns were not identified in our study, the previously reported characteristic changes of CXCL13-CSF levels in autoimmune and viral encephalitis may point to a meaningful marker of CNS immune activation, still warranting continued research [46, 48]. In AE, this could help identify patients experiencing immunologically active phases of disease and potentially guide decisions regarding immunotherapy [9, 49]. Similarly, in viral encephalitis, early CXCL13 increase in serum/CSF may assist in confirming a robust inflammatory response and help monitor the resolution of infection, though the overlap in levels across conditions underscores the need for cautious interpretation and combination with other diagnostic modalities [50, 51].

Finally, in our study we have observed a strong positive correlation between pleocytosis and CXCL13-CSF levels, both in LNB as in all analyzed patients with neuroinflammation which is similar to other data [38, 43, 44]. No such association was present in viral and autoimmune encephalitis, while these were reported in some other studies [38, 48]. On the other hand a positive correlation was confirmed between CXCL13 and protein levels in CSF when analyzed all patients, as well as, in LNB and AE cases separately. This correlation was reported in the past in regards to LNB, however, to our knowledge there was no such reports for AE [12].

The major limitation of the study is the relatively small study group. However, all analyzed patients are well clinically defined and represent diverse types of different neuroinflammation. A positive aspect of the study is a well-selected control group, which includes a comprehensive set of general CSF analysis results. Small sample size also makes it challenging to reliably interpret CXCL13 levels in CSF, especially given the lack of disease

specificity, absence of standardized cut-off values, variability in testing methods, and overlap with other neuroinflammatory or non-inflammatory conditions [22, 38, 52]. Addressing these limitations in the future will require larger studies and efforts to standardize assays and diagnostic thresholds.

In conclusions, we have confirmed the utility of CXCL13 measurements in CSF for LNB diagnosis. No specific patterns in CXCL13-CSF levels were associated with viral and autoimmune encephalitis. Nevertheless, CXCL13-CSF upregulations cannot be excluded in individual cases, thus the parameter should be used with precautions and only as supplementary diagnostic marker.

Conflict of interest disclosure

The authors declare that they have no competing interests.

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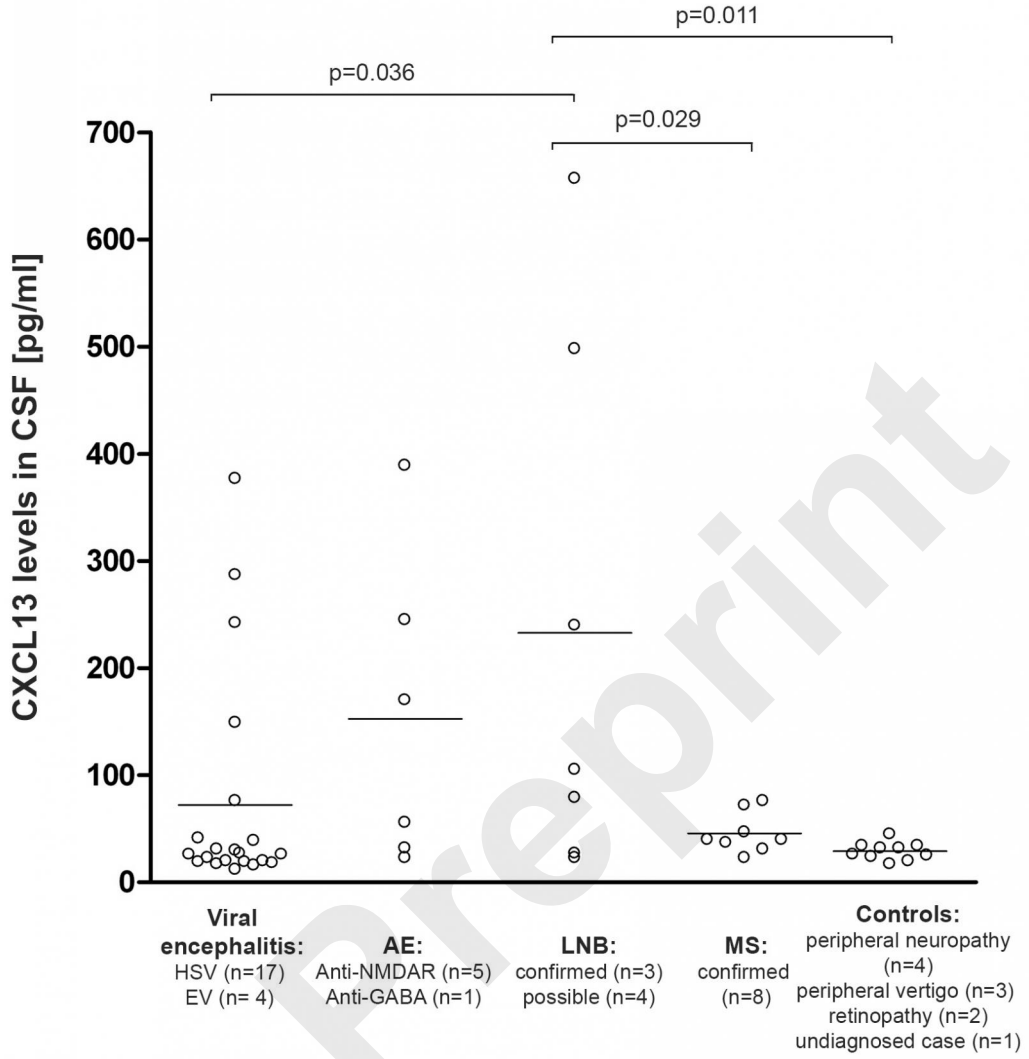
Figure Legends:

Figure 1. Concentration of CXCL13 in CSF (pg/ml) in patients with neuroinflammation ($n = 42$) and controls (ten subjects with absence of neuroinflammation). The patients were divided into the following groups: patients with viral encephalitis ($n = 21$), patients with autoimmune encephalitis (AE; $n = 6$), Lyme neuroborreliosis (LNB; $n = 7$) and multiple sclerosis (MS; $n = 8$) cases. Anova test (p=0.0093) was used to analyze the differences between the groups and all significant results were shown (p<0.05 in Tukey-Kramer test). Horizontal lines indicate mean value of CXCL13-CSF levels in each group.

Chemokine ligand 13 (CXCL13) attracts B lymphocytes by binding to its receptor CXCR5, guiding them into B-cell follicles within secondary lymphoid organs such as lymph nodes, spleen, and Peyer's patches.

CXCL13 has been reported to be a valuable diagnostic biomarker in Lyme neuroborreliosis (LNB).

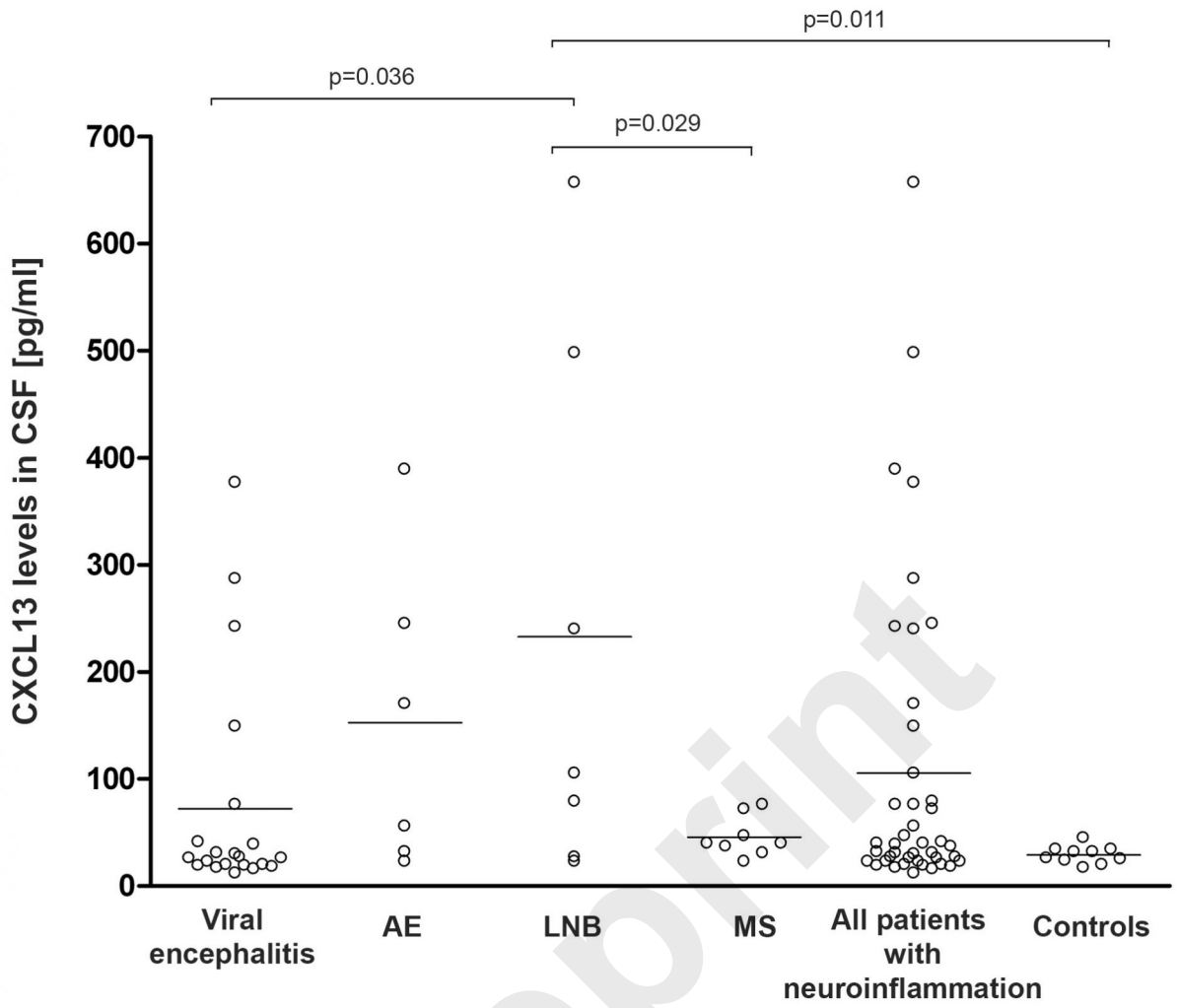
In the present study we have analyzed levels of CXCL13 in cerebrospinal fluid (CSF) in viral and autoimmune encephalitis (AE), as well as, in LNB and mulitple sclerosis (MS) patients to establish its possible diagnostic role in these types of encephalitis.



In conclusions, we have confirmed the utility of CXCL13 measurements in CSF for LNB diagnosis. No specific patterns in CXCL13-CSF levels were associated with viral and autoimmune encephalitis. Nevertheless, CXCL13-CSF upregulations cannot be excluded in individual cases, thus the parameter should be used with precautions and only as supplementary diagnostic marker.

Table 1. Clinical and laboratory data in patients with viral encephalitis, LNB – Lyme neuroborreliosis, MS – multiple sclerosis, AE – autoimmune encephalitis and controls – subjects without neuroinflammation.

	Viral encephalitis (n=21)	AE (n=6)	LNB (n=7)	MS (n=8)	All patients with neuroinflammation (n=42)	Controls (n=10)
Age in years; median (range)						
	36 (25-80)	41 (20-63)	39 (25-64)	34 (23-59)	36 (20-80)	30 (20-56)
CSF analysis:						
Cytosis in 1µl; mean (range)	87 (1-1225)	67 (2-208)	116 (1-602)	7 (3-13)	76 (1-1225)	2 (1-6)
% of lymphocytes; mean (range)	77 (43-97)	72 (12-100)	93 (83-100)	87 (84-91)	81 (12-100)	39 (14-80)
Protein [mg/dl]; mean (range)	55 (16-178)	61 (19-142)	102 (23-232)	30 (18-48)	60 (16-232)	34 (18-53)
Detected viruses, n (viral load in copies/ml)						
HSV	17 (60-341)	-	-	-	-	-
EV	4 (12-60)	-	1 (270)	-	-	-
AE antibody in CSF; n (%)						
Anti-NMDAR	-	5 (83.33)	-	-	-	-
Anti-GABA	-	1 (16.67)	-	-	-	-
CXCL13 levels in CSF [pg/ml]; mean (range)						
	72 (12-377)	153 (23-289)	233 (23-657)	46 (23-76)	105 (38-657)	29 (17-45)
Number of patients with CXCL13 levels in CSF above 100 pg/ml (%)						
	4 (19)	3 (50)	4 (57)	0	11 (26)	0


p=0.011
p=0.036
p=0.029
CXCL13 levels in CSF [pg/ml]
700
600
500
400
300
200
100
0
Viral encephalitis
AE
LNB
MS
All patients with neuroinflammation
Controls