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CXCL13: A Prognostic Marker in Multiple Sclerosis

By: Carolina Havervall
Supervisor: Prof. Fredrik Piehl

södertörns högskola
SÖDERTÖRN UNIVERSITY

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In the demyelinating autoimmune disease multiple sclerosis (MS) there is a great need for validated prognostic biomarkers that can give information about both prognosis and disease course. So far only clinical parameters have been shown to predict future outcome. CXCL13 is a potent B cell chemoattractant that has been suggested to be a potential biomarker candidate. The aim of this study was to investigate the usefulness of CXCL13 as a prognostic biomarker for MS.

Clinical, paraclinical, laboratory and MRI data about a large group of MS patients and controls were collected. CXCL13 levels in cerebrospinal fluid (CSF) samples from these patients were determined by standard enzymelinked immunosorbent assay (ELISA).

In general CXCL13 were increased in CSF in MS, especially in relapsing-remitting MS during relapses, i.e. with ongoing inflammations in the central nervous system. CXCL13 is a good candidate prognostic marker for MS, since newly diagnosed MS with high CXCL13 levels showed worsened disease course within five years. Most importantly, MS conversion occurred in higher rate in possible MS patients with high concentrations of CXCL13 in CSF, and in a shorter time point. This observation may support an early treatment decision in these patients.

In conclusion, this study provides support for an association between CXCL13 levels in the CSF and later development of disease severity in MS.

Key words: *CXCL13, multiple sclerosis, cerebrospinal fluid, prognostic marker, biomarker*

"The brain is not an organ. The brain is simply you."

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1 Introduction

1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is an autoimmune inflammatory demyelinating disease of the central nervous system (CNS) [1]. The CNS includes the brain and spinal cord and together with the peripheral nervous system (PNS) it constitutes our nervous system [2]. Loss of CNS tissue integrity is associated with microglial activation, cytokine production, gliosis, i.e. proliferation of astrocytes in damaged areas of CNS and infiltration of leukocytes in the brain [3, 4].

MS causes inflammation in the CNS with subsequent neurodegeneration, resulting in a variable degree of neurological handicap depending on extent and location of the damage. MS has extremely heterogeneous disease course, ranging from the benign, with minimal disability after 10-20 years, to the most severe, with major disability within a few year [5].

Autoimmune disorders arise when physiological tolerance to ”self” antigens is lost [6], i.e. in MS myelin antigen. Modifications of local innate immune response results in changes in the inflammatory milieu in CNS [7]. However, this does not mean that the immune system is the initiating factor of the disease. Many trigger factors have been suggested for explaining the development of MS and most likely a mix of these, i.e. genetics and environmental factors, can be the answer [8, 9].

MS disease leads to demyelination, tissue damage, loss of neurons and oligodendrocytes, gliosis, remyelination and inflammation involving B cells, T cells, macrophages and activated microglia [9]. High concentrations of antibodies, secreted by B cells, are found in the brain and CSF of patients with MS **[10]** and B cells are shown to be crucially involved in the pathogenesis of MS [11, 12].

At the moment the prevalence of MS in Sweden is approximately 140/100 000 citizen [13].

1.1.1 Treatments

MS was recognized as a separate disease in the late 1800s [14] and for long there has not been no effective treatments. However, since the mid 90-ties several immunomodulatory drugs have been available to treat patients in the subgroup relapsing-remitting MS (RRMS) [14]. The therapeutic goal is to avoid disease progression. Thus, broken nerve connections are in most cases irreversible, resulting in a variable degree of neurological handicaps.

1.1.2 The brain

A nerve cell consists of cell body, axon and dendrites. By release of neurotransmitters in the synaptic cleft in the end of the axon, signals diffuse across the next cell, making up an enormous network of the brain. For faster signaling myelin, consisting of a type of gliacells known as oligodendrocytes, surrounds the axons. These myelin layers are the main focus of attack by leukocytes in MS. In the brain, the nerve cell bodies are located in the outer part of the brain, referred to as the grey matter or the cortex, while the white matter consists of the extremely long nerve outgrowths. The vertebrate brain has three layers cover known as meninges and below these the cerebrospinal fluid (CSF) is gathered in the ventricles. CSF surrounds, flows, permeates, and forms an integral component of the brain and spinal cord [15, 16]. CSF gives mechanical protection, compensates for changes in the intracranial pressure and provides circulation of metabolites, nutrients, toxins and signaling substances [17]. The CSF is totally exchange three to four times a day in the adult [18, 19].

Another type of gliacells are the microglia. Microglia are the immune cells of the brain since leukocytes, macrophages and other immune cells present in the peripheral blood are separated from the CSF by the blood-brain barrier (BBB) [20]. These cells are not allowed in CSF. However, in MS, leukocytes can infiltrate CSF due to leaks in the BBB [10, 21, 22, 23]. BBB is an endothelial barrier maintaining homeostasis in CSF [24]. The endothelial cells are connected with tight junctions and control transport between cerebral capillaries and neuronal tissue [24].

1.1.3 Diagnosis

Laboratory data, magnetic resonance imaging (MRI) and clinical data are equally regarded when diagnosing MS.

1.1.3.1 Laboratory data

CSF samples of MS patients collected by lumbar puncture (LP), i.e. tapping of the fluid surrounding the spinal cord, provide important diagnostic data by telling about presence of immunoglobulin gamma (IgG), oligoclonal bands (OCB), mononuclear cell quantity, albumin quotient etc. in the CSF. Serum samples are not good for diagnosing MS since periphery blood is separated from CSF by the BBB. However, several studies have indicates increased levels of immune cells even in blood in MS and other inflammatory diseases [25, 26]. The mainly compartments of normal CSF are water, glucose, proteins (primarily albumin), immunoglobulins and a very low numbers of mononuclear cells are the main compartments of normal [27].

1.1.3.1.1 Immunoglobulin gamma

IgG are antibody molecules synthesized by B cells and they are presented in high concentrations in the brain and CSF in patients with MS [10, 28, 29, 30]. By measuring levels in the CSF of IgG and albumin protein, a standard known as IgG-index is obtained, validating the relative ratio of IgG to albumin. IgG-index is standard praxis in diagnosing MS today.

1.1.3.1.2 Oligoclonal bands

Bands of IgG molecules, i.e. OCB, are measured in CSF of MS patients. Since presence of these bands are highly characteristic for MS [31, 32]. IgG-index and OCB provides information about leak of the BBB [33] and intrathecal production of antibodies in CSF [34]. Under normal conditions there is no intrathecal production of immunoglobulins in CSF [35]. Thus, IgG-index and OCB are important aspects of inflammation apart from inflammatory cell numbers. However, studies indicate that all MS patients share the same clinical outcome, despite presence of OCB [36]. The disease lacks a god diagnostic biomarker and totally lacks any prognostic marker, except for clinical propositions.

1.1.3.1.3 Mononuclear cells

There is an association of myelin destruction and infiltration of T and B leukocytes and macrophages in the CSF in MS [37, 38]. Therefore, CSF mononuclear cell numbers provide important information about ongoing inflammations in the CNS.

1.1.3.2 Magnetic resonance imagining

MRI reflects disruption of the BBB with associated infiltrations of leukocytes from the blood [39]. MRI lesions, most often found in the white matter areas of the brain, indicate ongoing inflammation or scars, *scleros*, i.e. old inflammations [40]. The lesions are visualized due to changes in density of water molecules in areas of inflammation. Lesions are seen as white spots on black background.

However, the correlation between number, location and quantity of MRI lesions, and patient symptoms or degree of disease is weak [41, 42]. This clinical / radiological paradox complicates the diagnosing of the MS. Individuals with no sign of inflammation, when regarded as MRI lesions, can have extremely severe disease course showing many clinical symptoms and vice verse.

1.1.3.3 Clinical data

The diagnosis and subtyping of MS follows McDonald criteria [43]. McDonald focus on dissemination in time and space. MS diagnosis is fulfilled when two clinical episodes suggestive of MS, separated in time and space, are presented by clinical or paraclinical data [43]. The expanded disability status scale (EDSS) [44] is used during diagnosis for describing degree of neurological handicap in patients, as a result of damaged neurons and CNS inflammation. EDSS is an important measurement of clinical disease severity used in pharmaceutical trials, but also in routine clinical praxis. The higher EDSS numbers, in the 0-10 scale, represents a more severe degree of disease. The clinical findings regarding patient history and neurological examination is still the most important in diagnosing MS.

1.1.4 Disease course

MS can be divided into four subgroups; relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS) and clinically isolated syndrome (CIS).

1.1.4.1 Clinically isolated syndrome

CIS is pre stage for MS, describing individuals only fulfilling the diagnosis criteria in space, however, not in time, i.e. one clinical episode indicative of MS has occurred [45]. A majority of CIS patients convert to RRMS within a mean of 1.7 years [45].It has been demonstrated that early treatment initiation in CIS patients is effective in delaying conversion to MS [46, 47, 48]. This is of high importance, as the response to therapy is unpredictable at its initiation.

1.1.4.2 Relapsing-remitting multiple sclerosis

In most cases MS starts with a relapsing-remitting disease course, with shifting periods with and without clinical relapses, in majority of the cases (80-90%) [49]. Relapses are clinically detectable and depending on the location of the neuroinflammation completely different symptoms occur. The most common symptoms are sensory loss, loss of vision, muscle weakness, balance disturbance and ataxia [50]. RRMS is driven by inflammation and axonal loss can happen already in a early stage of disease [51, 52].

1.1.4.3 Progressive multiple sclerosis

The two distinct forms of progressive MS; primary progressive MS (PPMS) and secondary progressive MS (SPMS), differs in some aspects. Thus, PPMS patients display fewer inflammatory cells, more pronounced oligodendrocytes loss and axonal reduction [53, 54].

RRMS can over time convert to a stable progression of neurological defects, diagnosed SPMS [55]. The conversion usually takes place after 10 years of RRMS onset, in 50% of RRMS patients [56]. However, the disease course can already from onset increase continuously with no repairments. This happens in approximately 15% of all MS patients and is diagnosed PPMS [57].

1.1.5 Genetics

There is solid evidence for a genetic influence in MS [58]. The most important genes associated with increased risk for disease development are human leukocyte antigen (HLA)-DRB1 [59, 60, 61, 62], HLA-A [63], METTL1 [60], CYP27B1 [60], CD58 [59], HLA-B [60], TNFRSF1A [60], IL2RA [59, 62], IL7R, IL2R, CLEC16A, CD58, EVI5-RPL5 and VAV1 [59, 60, 63, 64, 65, 66, 67] and there are more constantly being evaluated for confirmation or rejection. The high amount of important genes maybe indicate that each of those contribute only a modest effect [68]. The heterogeneous genetic may also be explained by studies demonstrating heterogeneity in the initial immunological patterns of demyelination in MS [69]. Finding the causative genes could reveal key pathways influencing the disease [67].

An animal model of MS, known as experimental autoimmune encephalomyelitis (EAE), was established 80 years ago [70]. EAE is a useful tool for understanding disease mechanism and detecting genetic control of neuroinflammation [71, 72, 73, 74]. For example a region on rat chromosome 9 has been shown to regulate EAE [67].

1.1.6 Environmental factors

Vitamin D deficiency [75], low sunlight exposure [76] and cigarette smoking [77, 78] are suggested to be involved in development of MS. Involvement of common childhood infections, e.g. Epstein-Barr virus, have also been introduced as possible factors [79, 80]. However, there are also opposite theories rejecting this [81, 82].

1.2 Biomarkers

A biological marker or biomarker is "*a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic progresses, or pharmacologic responses to the therapeutic intervention*" [83]**.** The ideal biomarker should be reproducible, reliable, simple to use and non-invasive [84]. Several types of biomarkers, all with different purposes; diagnostic, prognostic, estimating hereditary risk or evaluating response to treatments, are of where importance when studying diseases.

A validated prognostic biomarker for MS informing about prognosis and disease course, is of paramount importance. So far only clinical parameters, such as degree of restitution of function after first bout, relapse frequency the first year and age at onset, have been shown to predict future outcome [5].

Salzer et. al. 2009, showed that neurofilament light in CSF is a good candidate for a prognostic biomarker for MS [85]. However, other diseases also showed significantly increased concentrations of neurofilament light in CSF [86].

Another important question is whether all MS patients really need lifelong immunotherapy and may drug holidays be introduced based on prognostic facts.

1.2.1 CXCL13

B cells are known to be crucially involved in the pathogenesis of MS [11, 12] and leukocytes have been found in MRI lesions, i.e. areas of inflammation [9]. Leukocytes express chemokine receptors and leukocyte recruitment is tightly mediated by specific adhesions molecules, known as chemokines [87].

CXCL13, also known as B lymphocyte chemoattractant (BLC) or B Cell-Attracting chemokine 1 (BCA-1) [88, 89] is a small cytokine strongly attracting B cells and in small numbers T cells and macrophages [90]. CXCL13 belongs to the CXC chemokine family. In CNS CXCL13 is expressed by cells in perivascula, inflammatory lesions and scattered parenchymal cells [91]. CXCL13 and its receptor CXCR5, also known as Burkitt´s lymphoma receptor 1 (BLR-1), are expressed by almost all B cells in CSF from patients with MS [92, 93]. Likewise, EAE have also reported high amount of CXCL13 in CNS of mice with RRMS and SPMS like inflammations [94]. Patients with viral and bacterial infections, e.g. Lyme borreliosis, have also shown increased levels of CXCL13 in CSF [95, 96, 97].

1.3 Laboratory methods

1.3.1 Enzymelinked immunosorbent assay

Enzymelinked immunosorbent assay (ELISA) is a molecular biologic assay designed to measure antibody or antigen levels in e.g. serum, plasma, saliva and cell cultures [98]. A monoclonal antibody specific for an antigen is pre-coated onto a 96-hold microplate. Standards and samples are pipetted into the wells and any antigen present is bounded by the immobilized antibody. Unbound substances are washed away and an enzyme-linked monoclonal antibody specific for the antigen is added to the wells. Another wash follows to wash away unbound antibody-enzym reagents. Substrate solution is added to the wells and colour developed in proportion to the amount of antigen bound in the initial step. Development of the colour is stopped and finally the intensity of the colour is measured [98].

2 Hypothesis

The B cell chemoattractant CXCL13 is a good candidate for being a prognostic marker for MS.

3 Aim

The aim of this study was to collect data about a large group of MS patients, CIS patients and controls, from different databases, to measure levels of CXCL13 in CSF samples from these patients, ant to investigate whether there is a correlation between CXCL13 levels in CSF patients and MS disease activity.

4 Materials

4.1 Databases

Take Care (Journal System of Karolinska University Hospital)

Swedish MS-registry (www.msreg.net)

Stockholms Läns Landsting (SLL) electronic clinical records database

4.2 Laboratory kit

ELISA R&D Systems Quantikine Kit Human CXCL13/BLC/BCA-1

4.3 Samples

Cerebrospinal fluid samples of 387 MS patients:

- 322 relapsing-remitting MS (RRMS)
- 40 secondary progressive MS (SPMS)
- 24 primary progressive MS (PPMS)
- 87 clinically isolated syndrome (CIS)

Cerebrospinal fluid samples of 340 controls:

- 181 other neurological disease (OND)
- 145 inflammatory other neurological disease (iOND)
- 24 viral/bacterial central nervous system infections
- 14 healthy control (HC)

4.4 Program for statistical analysis

GraphPad Prism 3.0, San Diego, CA

5 Methods

5.1 Collecting data

Clinical, paraclinical, laboratory and MRI data on 387 MS patients (322 RRMS, 40 SPMS, 24 PPMS, 87 CIS) and 340 controls (181 OND, 145 iOND, 24 viral/bacterial infections, 14 HC) were collected from three databases; Take Care (Journal System of Karolinska University Hospital), the Swedish MS-register (www.msreg.net) and SLL (Stockholms Läns Landsting). In point of crucial data missing or being unclear, neurologist Fredrik Piehl updated patients' journals. The majority of data was gathered from Take Care and SLL.

The following parameters were included as potential early predictors of disability in MS

- CSF cell counts, i.e. mononuclear cell quantity
- CSF immunoglobulin G index
- CSF oligoclonal bands
- Latest diagnosis
- Latest expanded disability status scale (EDSS)
- EDSS date
- Number of relapses
- Year of onset
- Magnetic resonance imaging lesions

Following parameters were included in the raw data file, as parameters for future studies on biomarkers in MS

- CSF IgG
- CSF albumin quotient
- Plasma IgG

- Sex
- Age
- Ethnicity
- Heredity
- Haplotype
- Immunomodulating treatments

5.2 Enzymelinked immunosorbent assay (ELISA)

CXCL13 levels in cerebrospinal fluid were determined by standard enzymelinked immunosorbent assay (ELISA) R&D Systems Quantikine Kit, according to the manufacturer's instructions (R&D Systems), on cerebrospinal fluid samples from 387 MS patients and 340 controls collected in association with patients visiting the neurological clinic, Karolinska University Hospital, Solna or Huddinge.

CSF had been drawn collected in plastic tubes, centrifuged and stored as frozen cell pellet and cell-free CSF, respectively, at -80°C until analysis. Lumbar puncture (LP) and CSF sample preparation had been done by doctors, nurses and laboratory personal at the neurological clinic, Karolinska University Hospital, Solna or Huddinge.

Due to the fact that lumbar puncture is an invasive procedure, with risk of attracting head ache, controls mainly consisted of samples obtained from patients investigated for possible neurological disease (iOND, OND). However, also 14 healthy controls (HC) were studied.

5.3 Statistical analysis

Differences in relative levels of CXCL in CSF in MS and controls (iOND, OND and HC), and correlation analysis were tested for significance with the non-parametric Kruskal-Wallis test and Dunn´s correlation for multiple testing (GraphPad Prism 3.0, San Diego, CA).

6 Results

6.1 CXCL13 in cerebrospinal fluid in MS and controls

Comparing levels of CXCL13 in CSF with ELISA, indicated that CSF CXCL13 were overall increased in MS (including CIS) compared to control groups (figure 1A), except for viral/bacterial central nervous system infections cohort (figure 1B). Among different subgroups of MS (RRMS, PPMS, SPMS and CIS) highest levels of CXCL13 CSF were shown during clinical relapses, i.e. inflammations in the central nervous system (figure 1C).

6.2 CXCL13 in cerebrospinal fluid versus number of relapses during five years, EDSS and number of lesions in RRMS

RRMS cohort was used for analyzes between CSF CXCL13 and MS disease activity and progression. CSF CXCL13 in 52 RRMS patients, followed up five years from disease onset, indicated an association between high levels of CXCL13 in CSF and high EDSS (figure 2A). Furthermore, in analyzes including 322 RRMS patients, correlations between CSF CXCL13, EDSS and MRI lesions were obtained. The results showed that worsen disease outcome and larger quantity of MRI lesions were associated with higher amounts of CXCL13 in CSF (figure 2B, 2C). Summarized, CSF CXCL13 levels followed MRI lesion quantity and MS disease worsening (EDSS and relapse quantity) when a period of five years from onset was regarded.

6.3 CXCL13, cell counts and IgG-index in cerebrospinal fluid in MS

CSF CXCL13 showed significant positive correlation with CSF cell counts and CSF IgG-index (figure 3A, 3B). High levels of CXCL13 were associated with high quantity of cell numbers and IgG-index, and vice verse. However as shown by the correlation coefficient, CXCL13 versus cell counts presented a slightly better correlation than CXCL13 versus IgG-index.

6.4 Cell counts and IgG-index in cerebrospinal fluid versus number of relapses during five years and EDSS in RRMS

A RRMS cohort with 52 patients, followed up for five years from disease onset, were used for analyzes considering relapses quantity. The RRMS cohort for EDSS and MRI lesion analyzes consisted of 322 RRMS patients. High CSF cell counts correlated with high relapse frequency (figure 4). However, no correlation with EDSS was shown (figure 5). Considering IgG-index, neither EDSS nor relapse frequency showed any correlation (figure 6, figure 7).

6.5 CXCL13 and oligoclonal bands in cerebrospinal fluid in MS and CIS

High CXCL13 levels in CSF were related to the presence of oligoclonal bands (OCB) in MS patients (including CIS) (figure 8).

6.6 CXCL13 in cerebrospinal fluid and conversion to MS

A possible correlation regarding CXCL13 in CSF and conversion to MS was examined in two different analyses, including and not including time limitation. With time excluded, disease development occurred in at a significantly higher rate in the CIS cohort with higher levels of CXCL13 in CSF (figure 9A). With time included, conversion to MS showed a trend towards a

correlation with CXCL13 (figure 9B). Summarized, CSF CXCL13 was related to MS conversion and trend to conversion time.

7 Discussion

In the demyelinating autoimmune disease MS there is a great need for a validated prognostic biomarkers that can give information about both prognosis and disease course. So far only clinical parameters, such as degree of restitution of function after first bout, relapse frequency the first year and age at onset, have been shown to predict future outcome [5]. CXCL13 is a potent B cell chemoattractant that has been suggested to be a potential biomarker candidate [99]. The aim of the present work was to investigate the usefulness of CXCL13 as a prognostic biomarker for MS.

The results obtained here demonstrated that MS cohort had higher levels of CXCL13 in CSF compared to CIS, i.e. possible MS patients who have had one relapse but the dissemination in time have not been fulfilled clinically (new relapse) or with MRI, and different control groups (inflammatory other neurological disease (iOND), other neurological disease (OND) and health control (HC)) (Figure 1A). However, patients suffering from viral and bacterial infections presented even higher levels of CXCL13 in CSF, than MS patients (Figure 1B). Earlier reports have shown increased CXCL13 concentration in CSF in patients with Lyme neuroborreliosis and other inflammatory diseases of the CNS [100, 101, 102]. This implies that CXCL13 cannot be used as a diagnostic marker for MS in relation to infectious conditions, but may still be used as a prognostic marker when comparing different subgroups of MS. Due to the fact that bacterial and viral infections raise the CXCL13 concentrations, relevant infectious conditions need to be excluded in the diagnostic work up of MS, before CXCL13 can be used as a prognostic marker. These analyzes are standard procedure in clinical praxis when diagnosing MS today.

The extremely high levels of CSF CXCL13 in bacterial and viral infections could be explained by these conditions being acute infections. Acute infections result in an acute increase in the release of chemokines to quickly attract immune cells to infected areas. Infections are often characterized by much greater increase in cell numbers, i.e. CSF pleocytosis, compared to MS and also with a mixed population of mono- and polynuclear cells compared to the pure mononuclear cell population seen in MS.

Correlation analysis of CXCL13 levels in CSF and CSF cell counts showed that these parameters are significantly correlated. However, it is important to keep in mind that in MS the high levels of CSF CXCL13 is a result of a chronic CNS inflammatory process. It is important to discriminate between inflammation caused by infections and autoimmunity as treatment differs.

Earlier studies have shown that CSF concentration of the B cell chemokine CXCL13 differs in patients with CIS, relapsing-remitting MS (RRMS), primary progressive MS (PPMS) and secondary progressive MS (SPMS) [99]. Thus, RRMS present the highest levels of CSF CXCL13. However, this study was performed in a limited number of patients and it is unclear if these results reflect true differences. My aim with the present work was to validate these findings in a much larger cohort. The results were largely similar with RRMS having the highest concentration of CXCL13 in CSF (Figure 1C), especially during periods of clinical relapses, probably as a reflection of a flare in ongoing inflammatory activity.

The RRMS cohort was used to analyze correlations between CSF CXCL13 and MS disease activity, indicated as number of relapses during five years, EDSS and MRI lesion quantity. These different parameters likely reflect partly different aspects of MS disease, i.e. inflammatory activity and the degree of damage to nervous system. Thus, disease activity and disease progression are two different parameters, where the former can be assessed by relapse frequency and number of MRI lesions and the latter by EDSS, showing degree of neurological disability. The RRMS cohort was chosen for these analyzes, since both these parameters are reflected and that it by far is the largest MS subgroup in this material.

CSF CXCL13 levels correlated to both MRI lesion number and MS disease worsening (EDSS and relapse frequency) during a period of five years from follow up (Figure 2A-C). Considering this CXCL13 may be used as a biomarker for autoimmune CNS inflammation. These results are of importance, since currently there is no prognostic marker for MS.

All MS treatments today act as immunomodulators, but does not reverse already acquired neurological disability, i.e. healing of already damaged nerve connections. The question of interest is to what degree CXCL13 at sampling can predict future disease activity as this can help with treatment decisions. Another relevant question is if CXCL13 levels can predict treatment response or even be used as a marker of treatment response. However, larger longitudinal studies with repeated CSF sampling will be needed to answer these questions.

CXCL13 is a chemoattractant for lymphocytes and especially B cells [90]. B lymphocytes synthesize IgG, i.e. antibody molecules. Analyzing IgG-index and OCB provides infor-

mation about intrathecal production of antibodies, which is one important aspect of inflammation apart from inflammatory cell numbers. In the present work CSF CXCL13 showed significant positive correlation with CSF cell counts and CSF IgG-index (Figure 3A-B). However, as shown by the correlation coefficient, CSF CXCL13 versus CSF cell counts presented a slightly better correlation than CSF CXCL13 versus CSF IgG-index.

CSF cell counts primarily reflect T cell quantity being the largest population in CSF, whereas, B cells and monocytes constitute much smaller proportions. It would be of interest to measure also B cell numbers in CSF, due to the fact that CXCL13 is a B cell chemoattractant **(ref)**. Flow cytometry is standard to differentiate T and B cells. However, this is difficult to do in CSF due to the fact that cell numbers are a 1000 fold lower than in blood. This is not performed in normal clinical praxis.

CXCL13 and CSF cell counts provided partly overlapping information on inflammatory disease activity in MS. Patients with high CXCL13 levels and high CSF cell numbers both showed increased risk for a worse disease outcome (when indicated as relapse frequency) (Figure 2A, 4). Patients with the highest numbers of relapses during five years from onset presumably had the highest inflammatory activity and vice verse. This is the largest study ever made on CSF cell counts in MS. This signifies that the result of CSF cell counts correlating with clinical disease activity also is novel, and constitutes an interesting subject for future analysis. Even if CSF cell counts correlated with relapse quantity, no correlation with EDSS was shown (Figure 5). Thus, for now these findings suggests that it is more relevant to use CXCL13 rather than cell counts, when predicting disease activity, since CXCL13 correlated with all parameters including EDSS, relapse frequency and MRI lesion quantity (Figure 2A-C). Another issue is that cell counts can be performed in different ways using automated machines or manually by using cell counts chambers. It is therefore more difficult to standardize than validating an ELISA, where standards can be used to validate the measurements.

Considering IgG-index, neither EDSS nor relapse frequency showed any association with risk for worse MS disease outcome (Figure 6, Figure 7). These possible associations were of interest to analyze due to the obtained results in Figure 3A and B, showing significant positive correlations between CXCL13, cell counts and IgG-index in CSF.

High CXCL13 levels in CSF were related to the presence of oligoclonal bands (OCB) in MS patients (including CIS) (Figure 8). The presence to OCB is believed to reflect an autoimmune inflammation, even if it may also occur as a post-infectious phenomenon.

However, the presence of OCB is not a good prognostic marker for disease activity, due to OCB being difficult to characterize. Furthermore, earlier studies have indicated that all MS patients share the same clinical outcome, despite presence of OCB.

CIS is a pre stage for MS and majority of patients diagnosed CIS convert to MS within a mean of 1.7 years [45]. A possible correlation regarding disease conversion and CXCL13 in CSF was of high importance for the present work. In the first analysis time to conversion was not regarded. With time excluded, disease development occurred in at a significantly higher rate in the CIS cohort with higher levels of CXCL13 in CSF (Figure 9A). Considering the fact that CIS patients usually convert to MS within a mean of 1.7 years I chose a time limit of 2 years for disease development in the second analysis. With time included, conversion to MS showed a trend towards a correlation with CXCL13 (Figure 9B). In summary, CSF CXCL13 was related to MS conversion and trend to conversion time.

In conclusion, this study showed that CXCL13 levels are elevated in MS patients displaying higher disease activity. Even if these findings need replication in other cohorts data so far suggest that CXCL13 can be used as a prognostic marker, especially in RRMS.

These results are of importance since they shed light on underlying disease processes in MS, where there is a great need of biomarkers. There have been prior reports on biomarkers reflecting inflammation and impact on neurons in MS. Xx et al 2009, showed that neurofilament light could be used as a prognostic biomarker for MS [85]. However, other diseases also showed significantly increased concentrations of neurofilament light in CSF [86], i.e. neurofilament light is unreliable as a diagnostic MS biomarker. The conclusion is that it may be difficult to find a single prognostic marker that reflects all different aspects of MS. Instead the use of several different biomarkers together (such as CSF CXCL13 and neurofilament light) could give reliable prognostic information on disease activity and risk of future disability in MS. An important use of such biomarkers would be to aid treatment decisions in MS.

In future work on prognostic and diagnostic biomarkers, response to treatments etc, the file containing data on MS patients and controls made here can be of great value, providing critical information for these important and valuable studies on MS.

Starting this work I hoped if gaining valuable results on CSF CXCL13 as a prognostic biomarker, analyzing levels of this chemokine may be done in hospitals all over the world. With this thought in mind I wanted to use an easy and eligible method for the ana-

lyze. A method that is available in laboratories worldwide. Therefore, as a method for measuring CXCL13 levels in CSF I chose enzymelinked immunosorbent assay (ELISA).

MS causes inflammation in the CNS with subsequent neurodegeneration, resulting in a variable degree of neurological handicaps. CXCL13 may be a potential biomarker on inflammation in MS. As inflammation is a treatable parameter, the disease pathway could be targeted, consequently leading to prevention of handicap.

In conclusion, this study provides support for an association between CXCL13 levels in the CSF and later development of disease severity in MS.

8 Conclusions

- In general CXCL13 is increased in CSF in MS, especially in RRMS during relapses.
- Levels of CSF CXCL13 correlates with CSF cell counts and CSF IgG-index.
- CXCL13 is a good candidate prognostic marker in MS, since newly diagnosed MS with high CXCL13 levels show worsened disease course within five years.
- Individuals with oligoclonal bands (OCB) in CSF show much higher levels of CXCL13 in CSF, compared to those without OCB.
- Most importantly, MS conversion occurs in higher rate in CIS patients with high concentrations of CXCL13 in CSF, and in a shorter time point. This observation may support an early treatment decision in these patients.
- These findings also support the use of CXCL13 as a biomarker in studies seeking to identify the role of genetic risk factors for the development of intrathecal inflammatory responses.

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11 Figures

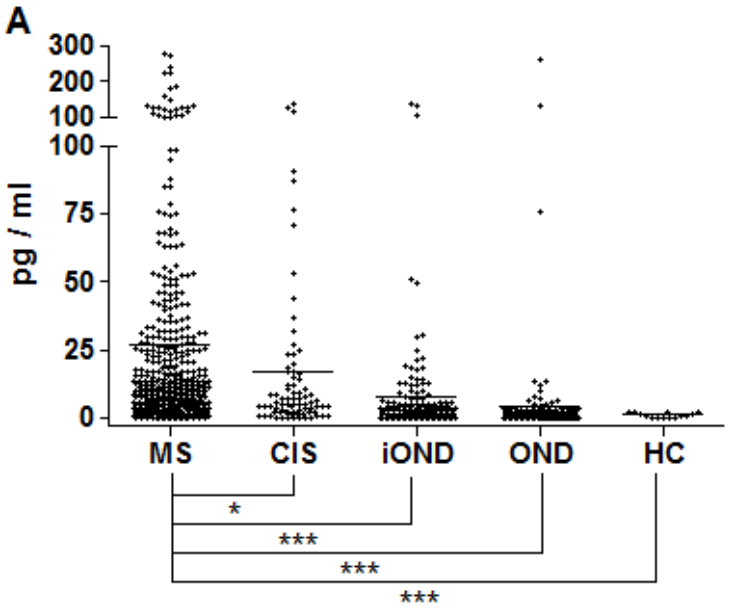

Figure 1A CXCL13 in CSF (all cohorts)

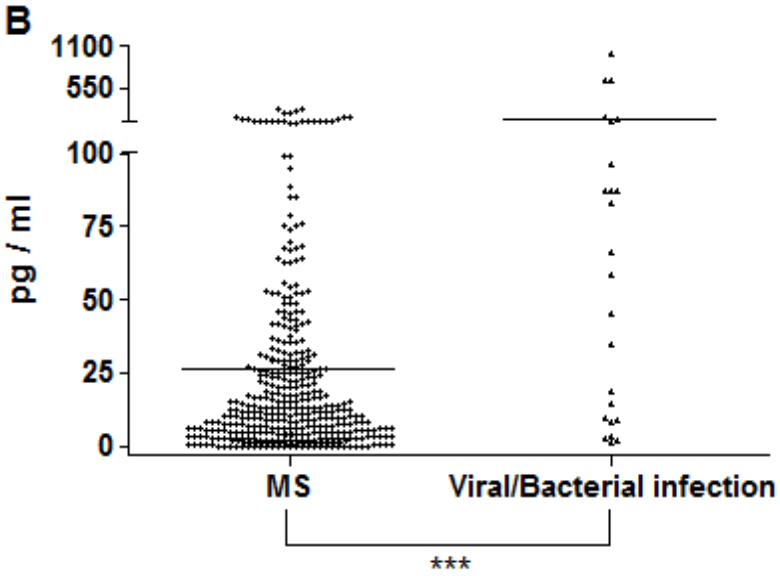

Figure 1B CXCL13 in CSF (all MS cohorts and viral/bacterial central nervous system infections cohort)

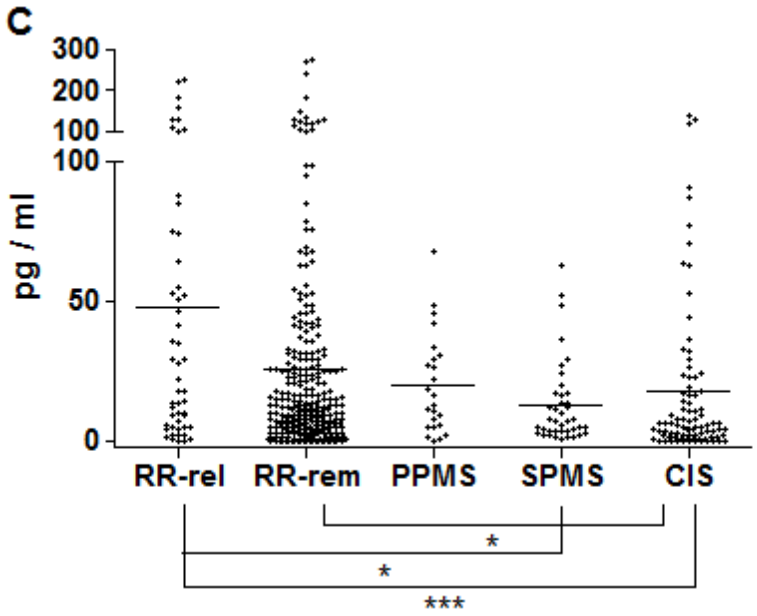

Figure 1C CXCL13 in CSF (all MS cohorts)

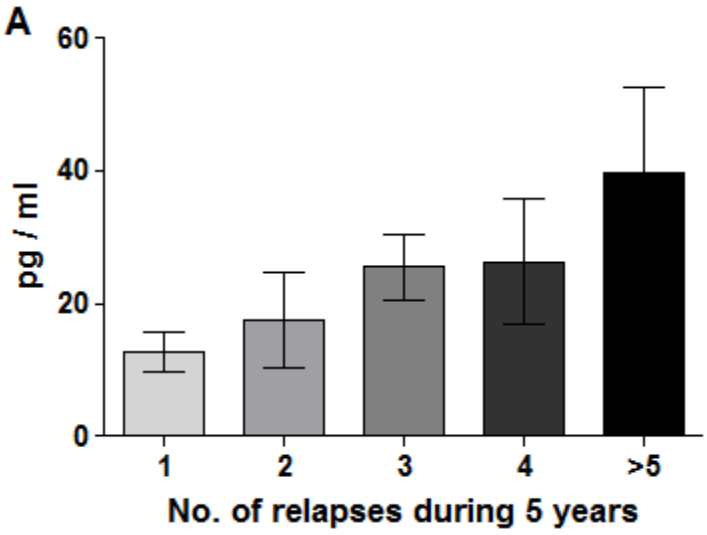

Figure 2A CSF CXCL13 versus number of relapses during 5 years (RRMS cohort)

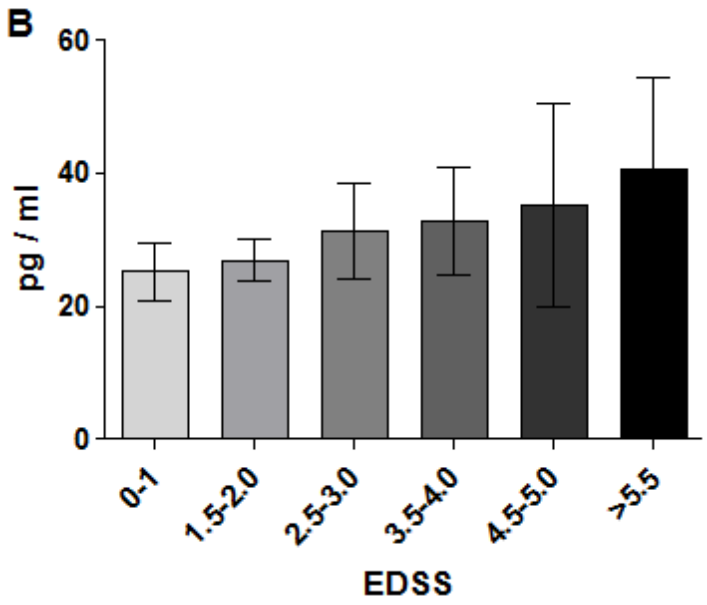

Figure 2B CSF CXCL13 versus EDSS (RRMS cohort)

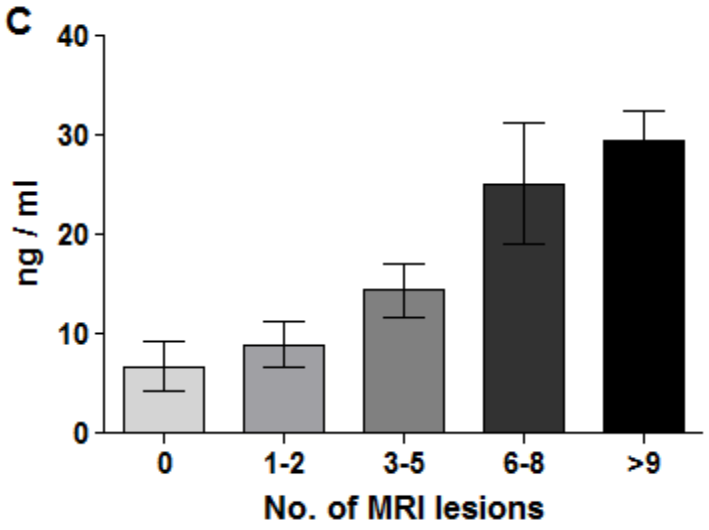

Figure 2C CSF CXCL13 versus number of lesions (RRMS cohort)

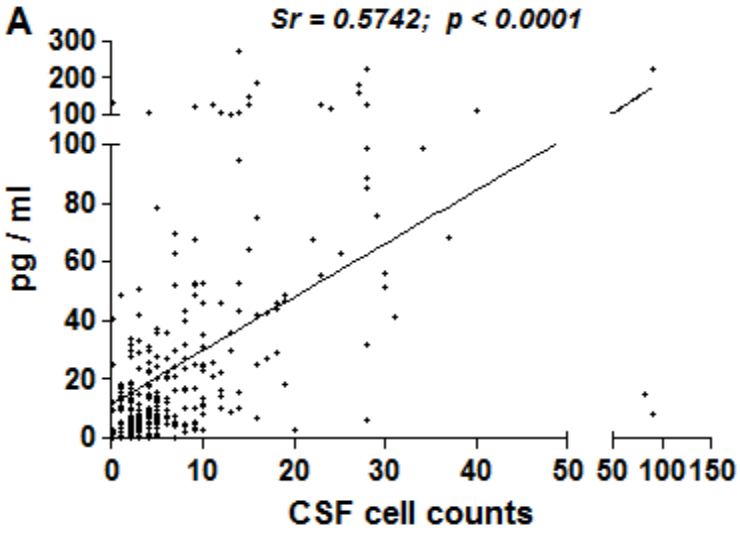

Figure 3A CSF CXCL13 versus CSF cell counts (all MS cohorts)

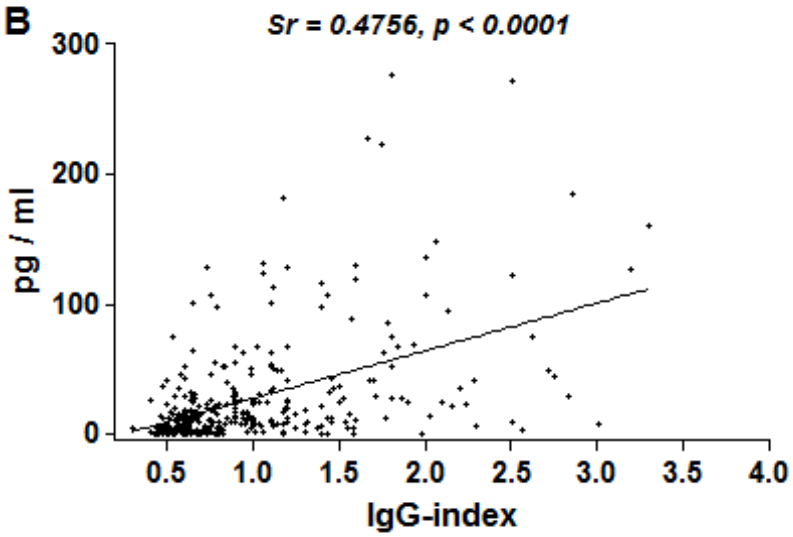

Figure 3B CSF CXCL13 versus CSF IgG-index (all MS cohorts)

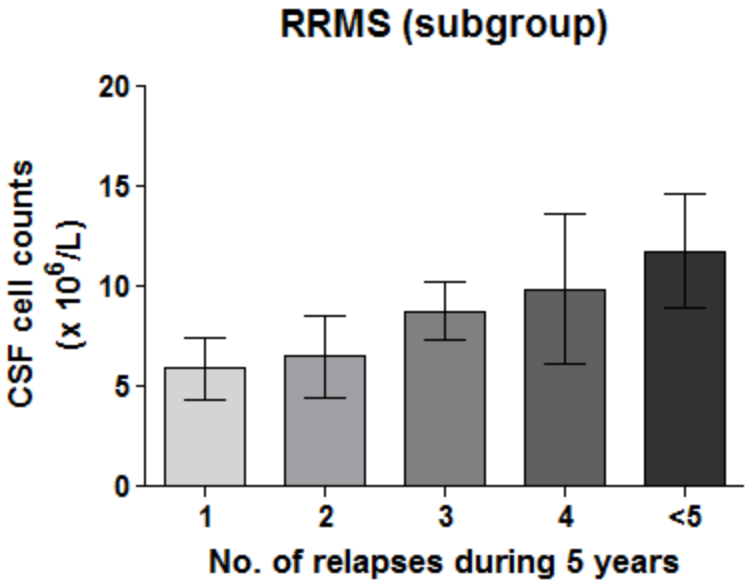

Figure 4 CSF cell counts versus number of relapses during 5 years (RRMS cohort)

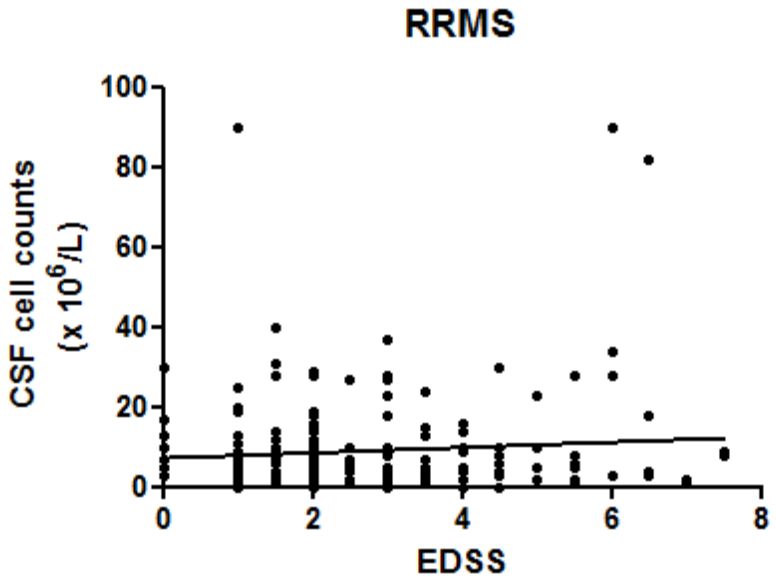

Figure 5 CSF cell counts versus EDSS (RRMS cohort)

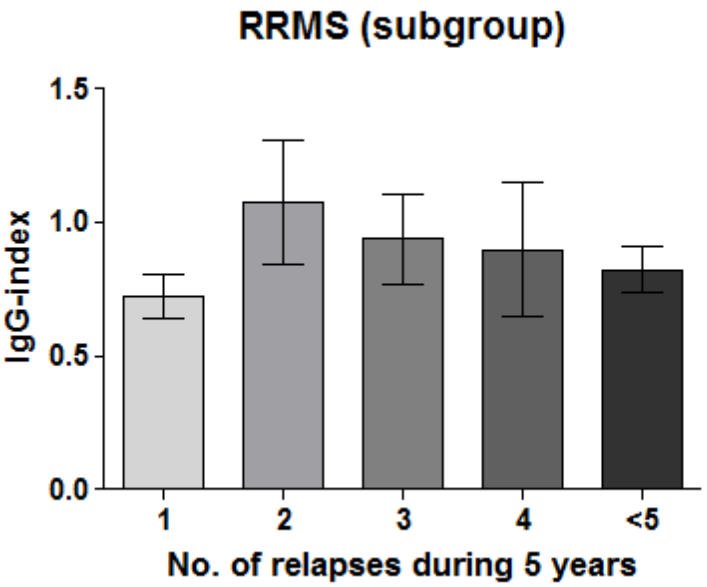

Figure 6 CSF IgG-index versus number of relapses during 5 years (RRMS cohort)

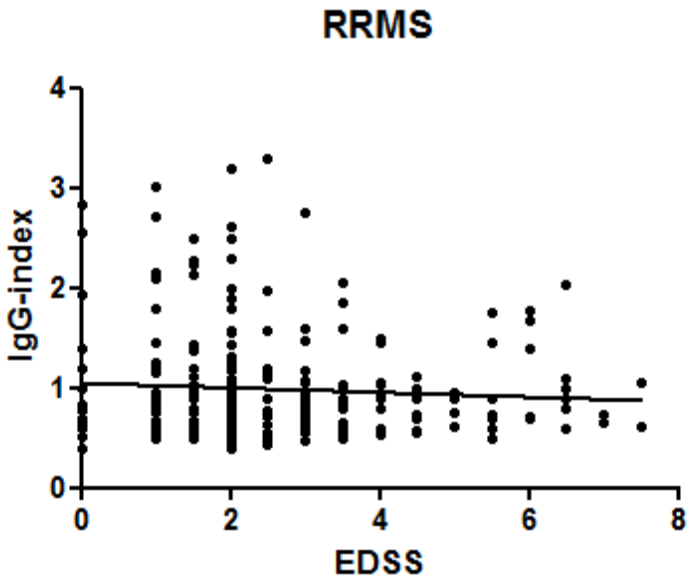

Figure 7 CSF IgG-index versus EDSS (RRMS cohort)

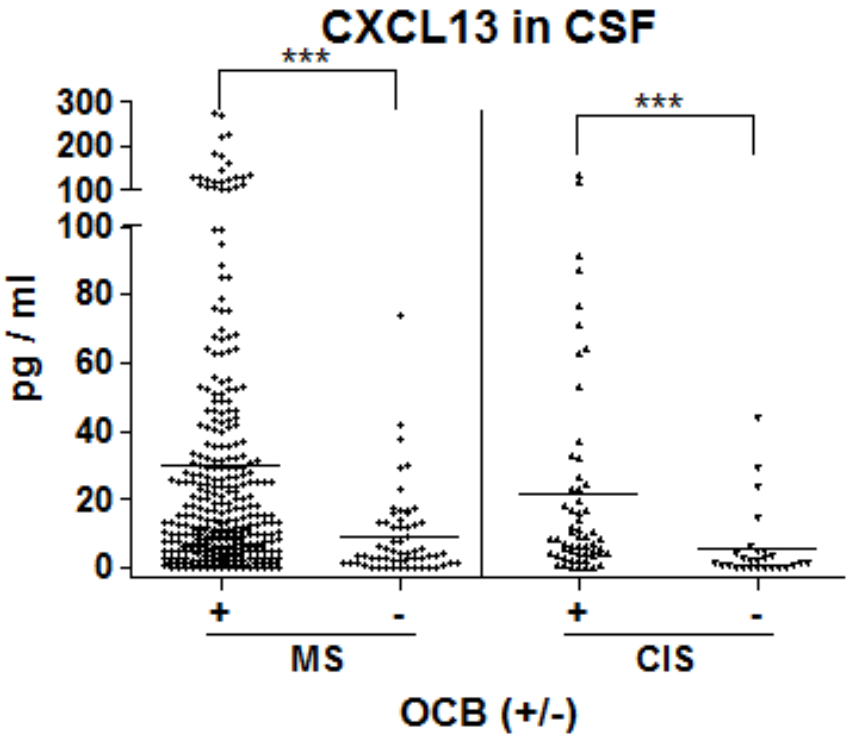

Figure 8 CSF CXCL13 versus oligoclonal bands (OCB (all MS cohort and CIS cohort)

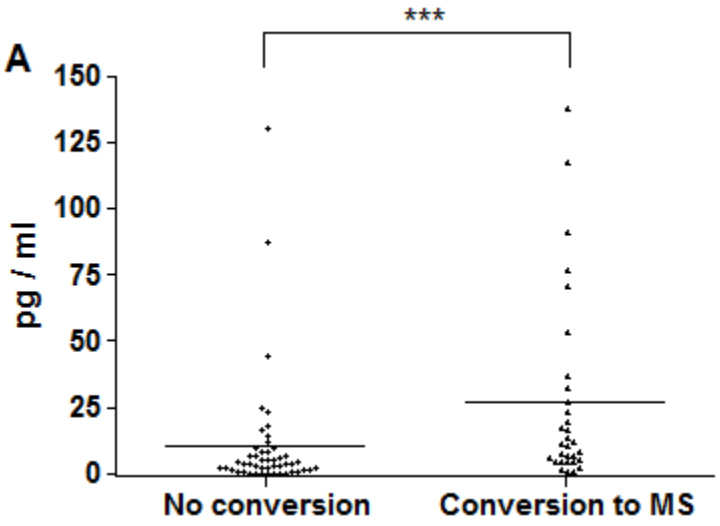

Figure 9A CSF CXCL13 versus conversion to MS (CIS cohort)

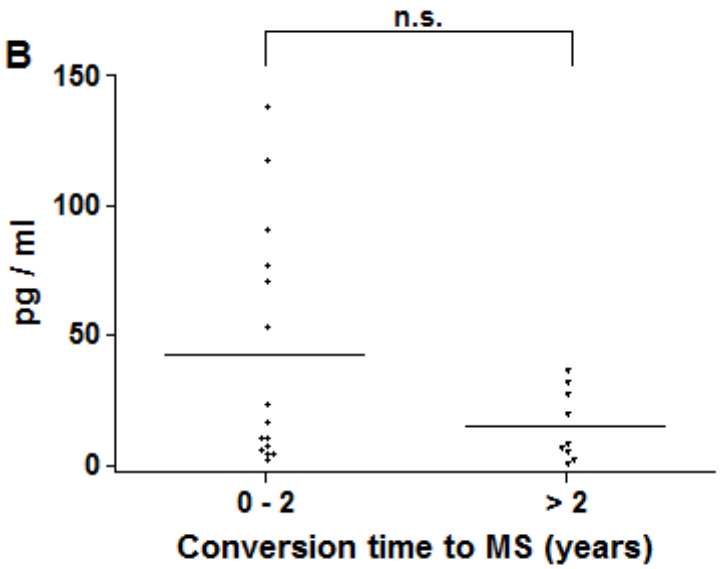

Figure 9B CXCL13 in CSF versus conversion time to MS (CIS cohort)