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High sensitivity and specificity of elevated cerebrospinal fluid kappa free light chains in suspected multiple sclerosis

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Abstract

Cerebrospinal fluid (CSF) analysis is routinely used in the diagnostic work-up of multiple sclerosis (MS), by detecting CSF-specific oligoclonal bands (OCB). More recently, several studies have reported CSF free light chains (FLC) as an alternative. We show that absolute CSF κFLC concentrations were highly sensitive - more than OCB testing - and specific for clinically isolated syndrome, relapsing remitting and primary progressive MS. Measurement of κFLC alone was sufficient. Our results suggest that CSF κFLC levels measured by nephelometry, if validated in a larger series, are a preferred test to OCB analysis in the diagnostic work-up of patients suspected of having MS.

Keywords

Multiple sclerosis; Oligoclonal bands; Free light chains

Highlights

CSF κ free light chains in multiple sclerosis are sensitively measured by nephelometry

CSF κ free light chains were highly specific for multiple sclerosis

Analysis of CSF κ free light chain concentration alone is a sufficient measurement

Abbreviations

FLC - free light chains; OCB - oligoclonal bands; CIS - clinically isolated syndrome; CSF - cerebrospinal fluid; RR-MS - relapsing remitting MS; PP-MS - primary progressive MS.

1. Introduction

The pathogenesis of multiple sclerosis (MS) involves inflammatory demyelination, thought to involve the activation of autoreactive T cells that in turn activate microglia and recruit macrophages, which cause demyelination and subsequent neurological damage (Disanto et al. , 2010; Nylander and Hafler, 2012). In addition, meningeal ectopic B cell follicles and plasmablasts in the CSF have been reported; these cells are also likely to contribute to the pathology of MS (Aloisi et al. , 2010; Meinl et al. , 2006). Despite refinements in MRI imaging protocols for the diagnosis of MS, the detection of intrathecal immunoglobulin, produced by these central nervous system resident B cells, continues to be an important component of the diagnostic pathway for MS (Katsavos and Anagnostouli, 2013; Tumani et al. , 2009). In addition to diagnostic information, particularly when MRI imaging is inconclusive, the presence of CSF specific OCB may provide prognostic information, for example in the prediction of conversion from clinically isolated syndrome (CIS), the first presenting neurological event, to relapsing remitting disease (RR-MS) (Villar et al. , 2012).

Traditionally, the assay for the presence of CSF immunoglobulin is performed using gel isoelectrofocusing and immunoblotting, allowing the visualisation of oligoclonal bands which may or may not be mirrored in the matched serum sample (Katsavos and Anagnostouli, 2013; Tumani et al., 2009). There are many difficulties with this test including requirements for a matched serum sample, manual handling, limited sensitivity and the potential for subjective interpretation. In an attempt to address these issues, a number of recent studies have employed the detection of CSF free light chains (FLC) as a measure of intrathecal immunoglobulin synthesis (Arneth and Birklein, 2009; Duranti et al. , 2013; Kaplan et al. , 2013; Presslauer et al. , 2008; Senel et al. , 2014; Villar et al., 2012). Light chains are

produced in excess during antibody formation and are secreted from the plasma cells/plasmablasts, with a serum half-life of 2-6 hours primarily through renal clearance (Solling, 1981). This pathway is absent for the CSF and FLC levels may therefore remain elevated for prolonged periods, to the point where even very small amounts of intrathecal FLC synthesis become detectable. The majority of studies on FLC in the CSF have shown an increased sensitivity and specificity for MS diagnosis as compared to traditional OCB detection (Arneth and Birklein, 2009; Duranti et al., 2013; Kaplan et al., 2013; Presslauer et al., 2008; Villar et al., 2012). However, the specific results vary according to the assay methodology, using either ELISA or nephelometry (Senel et al., 2014) which may have different sensitivities, and with the use of an index measure of FLC (Duranti et al., 2013; Messaoudani et al. , 2014; Presslauer et al., 2008), for example (CSF FLC/serum FLC)/(CSF albumin/serum albumin). In our study we have performed analysis of κ and λ FLC by nephelometry and demonstrate that the concentration of κFLC alone provides a high degree of sensitivity and specificity in MS.

2. Materials and Methods

2.1 Study Subjects

Ethical approval for the study was provided by the Human Biorepository Research Centre (HBRC), University of Birmingham. All subjects provided written informed consent to participate in this study. Between February 2011 and September 2013, matched serum and CSF samples were prospectively collected from 160 patients who underwent routine diagnostic lumbar puncture as elective cases on the Neurology day-case unit at the Queen Elizabeth Hospital, Birmingham (QEHB).

The following diagnostic groups were established: CIS, RR-MS, primary progressive MS (PP-MS), fulfilling the criteria of dissemination in space and time for diagnosis of MS according to recent criteria (Polman et al. , 2011), other neurological inflammatory diseases (ONID) and other neurological diseases (OND). CIS patients therefore had evidence of one attack, with objective clinical evidence of one lesion, but no evidence of dissemination in time (clinically or radiologically). The majority of MS patients were not receiving any disease-modifying treatment at time of lumbar puncture except one PP-MS patient who was taking methotrexate and one RR-MS patient who had been prescribed TYSABRI®, but had not received it in the 30 days prior to lumbar puncture. Two MS patients (one CIS and one RR-MS) had received a course of steroid therapy in the month prior to CSF collection. The details of the patient cohorts are given in Table 1.

2.2. Preparation of CSF and serum

Peripheral blood was collected in serum clotting tubes (Serum Sep Vacuette; Greiner BioOne, Germany) and allowed to clot for 20 min at room temperature before centrifugation (700*g*, 20 min), and stored at -80°C until analysis. A mean volume of 6 ml (range 2-24 ml) of

CSF was obtained by non-traumatic lumbar puncture. CSF was centrifuged (400*g*, 8 min) and the supernatant collected and frozen at -80^{o}C until analysis. The cell pellet was analyzed by flow cytometry (Cyan ADP High Performance flow cytometer; Beckman Coulter, High Wycombe, UK), with the addition of counting beads (Life Technologies, U.K.) to obtain a lymphocyte count. Only matched serum and CSF samples with adequate volume were included for analysis in the study.

2.3. Free light chain quantification

FLC concentrations were measured by nephelometry using the latex particle-enhanced, Freelite® κ and λ immunoassays (The Binding Site Group Ltd, Birmingham, U.K.) (Bradwell et al. , 2001) on a Dade-Behring BN ™ II Analyser, following the manufacturer's instructions. CSF samples were measured without dilution. Normal serum reference intervals were used: κFLC 3.3 – 19.4 mg/L and λFLC 5.7 – 26.3 mg/L with an assay sensitivity of < 1 mg/L (Katzmann et al. , 2002). Normal CSF reference intervals have not been defined. The limit of detection for the κFLC assay was 0.06 mg/L and 0.05 mg/L for the λFLC assay.

2.4 Data analysis

Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Statistical analysis used non parametric testing as specified.

3. Results

3.1 Measurement of FLC

The levels of CSF κFLC were significantly elevated in all 3 MS groups, CIS, PP-MS, and RR-MS, as compared to either the OND or ONID control groups (Fig. 1A, Table 2). By contrast, the levels of CSF λFLC, although elevated in all MS groups as compared to OND and ONID, failed to reach statistical significance in all cases; for the CIS and PP-MS groups there was a significant increase as compared to OND but not ONID. Interestingly, for the RR-MS group CSF λFLC levels were significantly elevated as compared to both OND and ONID groups (Fig. 1B). There were no significant differences in the levels of CSF κ or λ FLC between the MS groups, or serum κ or λ FLC between all groups (Fig. 1 C,D). The levels of CSF κFLC approached those detectable in the serum (Table 2).

Receiver operator characteristic (ROC) analysis revealed that measurement of CSF κFLC levels produced a high degree of sensitivity and specificity (Fig. 2 A,B), which was far superior to that with λFLC (Fig. 2 C,D), and not further enhanced by combining the values for CSF κ and λFLC (Fig. 2 E,F, Table 3), although the area under the curve was slightly elevated. For comparison of the measurement of κ and λFLC, as well as ratio measurements, the cut-off used was the level that resulted in a specificity of 98%. The characteristics of the ROC analysis were maintained for both the CIS alone (Fig. 2B,D,F), and when analysing all MS cohorts as a single group (Fig. 2A,C,E). A comparison of the CSF OCB and κFLC data for the MS group showed that there was a significant elevation of CSF κFLC in the CSF OCB positive *vs.* negative group (Fig. 2G). Importantly, 4/6 MS patients (2 CIS, 1 PP-MS, 1 RR-MS) that returned a negative CSF OCB test were positive for CSF κFLC when using a cut-off of 0.9 mg/L. A single RR-MS patient with a positive CSF OCB was very marginal at

this cut-off (0.95 mg/L) and one non-MS patient, that interestingly was also positive for the OCB test, was positive for CSF κFLC. There were two patients in the MS group (1 CIS, 1 RR-MS) that remained negative for both tests.

To account for potential influence of a breakdown of endothelial barriers in the CNS, total CSF protein was also measured. The ratio of CSF FLC to total protein, for both κ and λ FLC, showed very similar patterns and levels of significance to those using the CSF FLC measurement alone (Fig. 3A,B). Although ROC analysis demonstrated that the sensitivity and specificity were very high, they did not increase above the level for FLC alone (Fig. 3C, Table 3). A similar analysis of CSF to serum FLC ratios also showed similar results but again failed to enhance sensitivity or specificity (data not shown). There was no correlation with T2 lesion load (Fig. 4A), but there was a weak but significant correlation between CSF κFLC and the concentration of lymphocytes in the CSF (Fig. 4B).

Discussion

In this study we have demonstrated that nephelometric measurement of κFLC in CSF is highly sensitive and specific for CIS, PP-MS and RR-MS, classifying a number of individuals that were negative using traditional OCB testing. These data add to the growing number of studies that have shown the utility of CSF FLC measurement in the diagnosis of MS (Duranti et al., 2013; Kaplan et al., 2013; Messaoudani et al., 2014; Presslauer et al., 2008; Senel et al., 2014), and that this assay can be more sensitive than OCB testing. In addition we show that a κ FLC concentration measurement alone is sufficient to provide this high level of sensitivity and specificity in suspected MS. If validated in a larger series, this method will be an easier way of studying intrathecal immunoglobulin synthesis with a sensitivity and specificity at least comparable to that of oligoclonal IgG bands.

The samples were collected prospectively for diagnosis and we are currently awaiting follow-up data on the CIS (n=21) group to determine the frequency and rate of conversion to definitive MS. Given the concordance with the OCB data it is likely that the majority will relapse given enough time. Reports also suggest that FLC measurements might predict conversion to MS (Villar et al., 2012), so it will be important to determine if the FLC measurement alone is sufficient to predict conversion and/or relate to the frequency of future relapses and/or disease progression as this could provide prognostic information and potentially inform treatment approaches. There were a small number of outlier samples, which did not show the predicted levels of κFLC in the CSF, and it will be interesting to determine in follow-up if these ultimately were clinically misclassified or display a different disease phenotype.

The levels of CSF κFLC have been shown to relate to the number of CSF cells (Senel et al., 2014), and we were able to confirm that CSF κFLC levels in a combined cohort of CIS, PP-MS and RR-MS correlated with cellularity of the CSF. There may be a number of reasons for this association, which fall beyond the scope of this current study but are the subject of ongoing investigations. Firstly, the cellular infiltrate is known to include B cells and plasma cells/blasts (Meinl et al., 2006) and therefore the amount of secreted antibody and associated excess FLC may be proportional to the number of these cells in the CSF. The increased levels of CXCL13 in MS CSF (Khademi et al. , 2011), which were also shown to correlate with FLC levels (Senel et al., 2014) suggest that this may indeed be the case but this requires confirmation by direct measurement of plasma cell/blast number and FLC levels. Secondly, at least some of the FLC may be coming from tissue-resident plasma cells/blasts, and the FLC levels are therefore more likely to be related to the degree of tissue inflammation. This may be related to the time since the beginning of the CIS or relapse, as CSF samples are collected at varying time points after onset of the neurological episode.

The measurement of κFLC, rather than the whole antibody molecule, is currently based on the superior sensitivity of the FLC assay. The detection of different isotypes of Ig associated with κ (or λ) FLC might provide further diagnostic or prognostic information, but their utility in MS has yet to be determined. Although nephelometry is widely used within clinical diagnostic laboratories, an ELISA format could also be of benefit in some laboratories that do not have access to nephelometry equipment. However, a recent study by Senel et al., who used an ELISA based system, failed to show any increased sensitivity or specificity of the κFLC assay over traditional OCB testing (Senel et al., 2014). The very high values for both sensitivity and specificity in our study using nephelometry, allowed detection of FLC in some

OCB negative samples from CIS and RR-MS patients and combined with data from other recent studies using the same technique (Duranti et al., 2013; Presslauer et al., 2008; Villar et al., 2012), suggest that this nephelometric test for κFLC, even when used without an index measurement, provides an excellent test for the confirmation of CIS/MS. If validated in a larger series, the use of this more sensitive test would allow for early treatment with the increasingly large range of immunomodulatory treatments becoming available, and potentially provide prognostic information on the likelihood of early conversion to MS (Villar et al., 2012).

Figure legends

Figure 1. Free light chains (FLC) are elevated in the cerebrospinal fluid (CSF) from patients with multiple sclerosis (MS), but not other neurological diseases. The levels of κFLC and λFLC were measured in matched CSF (**A,B**) and peripheral blood serum samples (**C,D**), taken at the time of the diagnostic lumbar puncture. CIS, clinically isolated syndrome; PP-MS, primary-progressive MS; RR-MS, relapsing-remitting MS; OND, other neurological disease; ONID, other neurological inflammatory disease. Kruskal-Wallis multiple group comparison with Dunn's post test; **, $p<0.01$, ***, $p<0.001$. All other comparisons were not significant ($p>0.05$).

Figure 2. Receiver operator characteristic (ROC) analysis of cerebrospinal fluid (CSF) κ free light chain (FLC) reveals a high degree of sensitivity and specificity for multiple sclerosis (MS). ROC curves are shown for CSF κFLC (**A,B**), λFLC (**C,D**), and combined κ and λ FLC levels (**E,F**). The levels of CSF κFLC (cut-off dotted line of 0.9mg/L) in MS and non-MS groups subdivided according to a positive oligoclonal band (OCB) (**G).** CIS, clinically isolated syndrome.

Figure 3. Determining the ratio of cerebrospinal fluid (CSF) free light chain (FLC) to total protein does not increase sensitivity or specificity for multiple sclerosis (MS). The levels of CSF κ and λ FLC per unit protein in the CSF were determined for each group (**A,B**). CIS, clinically isolated syndrome; PP-MS, primary-progressive MS; RR-MS, relapsing-remitting MS; OND, other neurological disease; ONID, other neurological inflammatory disease. Kruskal-Wallis multiple group comparison with Dunn's post test; *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$. All other comparisons were not significant ($p>0.05$). Receiver operator characteristic curves are shown for CSF κFLC/protein (**C**).

Figure 4. Cerebrospinal fluid (CSF) κ free light chain (FLC) levels correlate with CSF lymphocyte cell number but not T2 lesion load. Correlations between CSF κFLC and T2 lesion load (**A**) and CSF cells/ml (**B**). CIS, clinically isolated syndrome; PP-MS, primary-progressive MS; RR-MS, relapsing-remitting MS. Spearman correlation.

Patient group	n	Gender M/F	Age median (range)	Diagnosis
Clinically-isolated syndrome (CIS)	21	6/15	37 (21-70)	
Primary-progressive MS (PP-MS)	11	5/6	51 (43-72)	
Relapsing-remitting MS (RR-MS)	21	3/18	40 (19-72)	
Other neurological disease (OND)	90	24/66	44.5 (17-85)	CSF pathways disease (4), Miscellaneous (12), Acute neurodegenerative disease (6), Undetermined (17), Functional (2), Headache syndrome (24), Degenerative (5), Idiopathic intracranial hypertension (8), Anxiety (3), Neuropathy (4), Vascular (1), Pain syndrome (4)
Other neurological inflammatory disease (ONID)	17	10/7	53 (24-83)	APS, Behçet's (2), Assymetric axonal neuropathy, Compressive myelopathy (2), Anti-MAG neuropathy, Neuroretinitis, Sjogrens syndrome, Idiopathic transverse myelitis, Aseptic meningitis, Inflammatory cervical myelopathy, Longitudinally Extensive Transverse Myelitis, Vasculitic sensory neuropathy, Neurosarcoidosis (2), Chronic inflammatory demyelinating polyneuropathy

Table 1. Patient characteristics.

Patient group	Sample type	κFLC (mg/L) (median; range)	λFLC (mg/L) (median; range)	κFLC/protein (median; range)
CIS[a]	CSF	8.08; 0.06-26.03	0.25; 0.10-9.49	22.52; 0.24-98.25
	Serum	12.88; 2.09-22.10	13.89; 7.73-55.54	ND[b]
PP-MS	CSF	7.56; 1.61-35.56	0.80; 0.08-3.96	24.39; 3.59-93.16
	Serum	12.34; 1.79-20.08	13.48; 2.22-17.56	ND
RR-MS	CSF	8.01; 0.14-29.12	1.29; 0.05-7.99	29.84; 0.63-87.08
	Serum	10.78; 6.42-22.23	11.35; 7.00-19.83	ND
OND	CSF	0.14; 0.06-0.79	0.08; 0.05-0.36	0.47; 0.10-2.83
	Serum	11.73; 1.59-74.52	13.20; 5.25-72.64	ND
ONID	CSF	0.25; 0.06-4.47	0.17; 0.05-0.71	0.54; 0.17-13.54
	Serum	13.77; 2.01-30.15	13.57; 5.78-45.86	ND

Table 2. CSF and serum free light chain (FLC) levels. [a]CIS, clinically isolated syndrome; PP-MS, primary-progressive MS; RR-MS, relapsing-remitting MS; OND, other neurological disease (OND); ONID, other neurological inflammatory disease. [b]ND; not determined.

Comparison	Sensitivity (%)	Specificity (%)	Area under curve	Cut-off used (mg/L)
CSF κFLC				
All MS vs. non-MS	96.2	98.1	0.971	0.87
CIS vs. non-MS	95.2	98.1	0.953	0.99
CSF λFLC				
All MS vs. non-MS	60.4	98.1	0.918	0.57
CIS vs. non-MS	33.3	98.1	0.904	0.59
CSF κ+λFLC				
All MS vs. non-MS	96.2	98.1	0.980	1.47
CIS vs. non-MS	95.4	98.1	0.981	1.54
CSF κFLC/protein				(ratio)
All MS vs. non-MS	94.2	98.1	0.975	2.98
CIS vs. non-MS	95.0	98.1	0.957	2.98

Table 3. Receiver operator characteristic analysis of CSF free light chain (FLC) levels.

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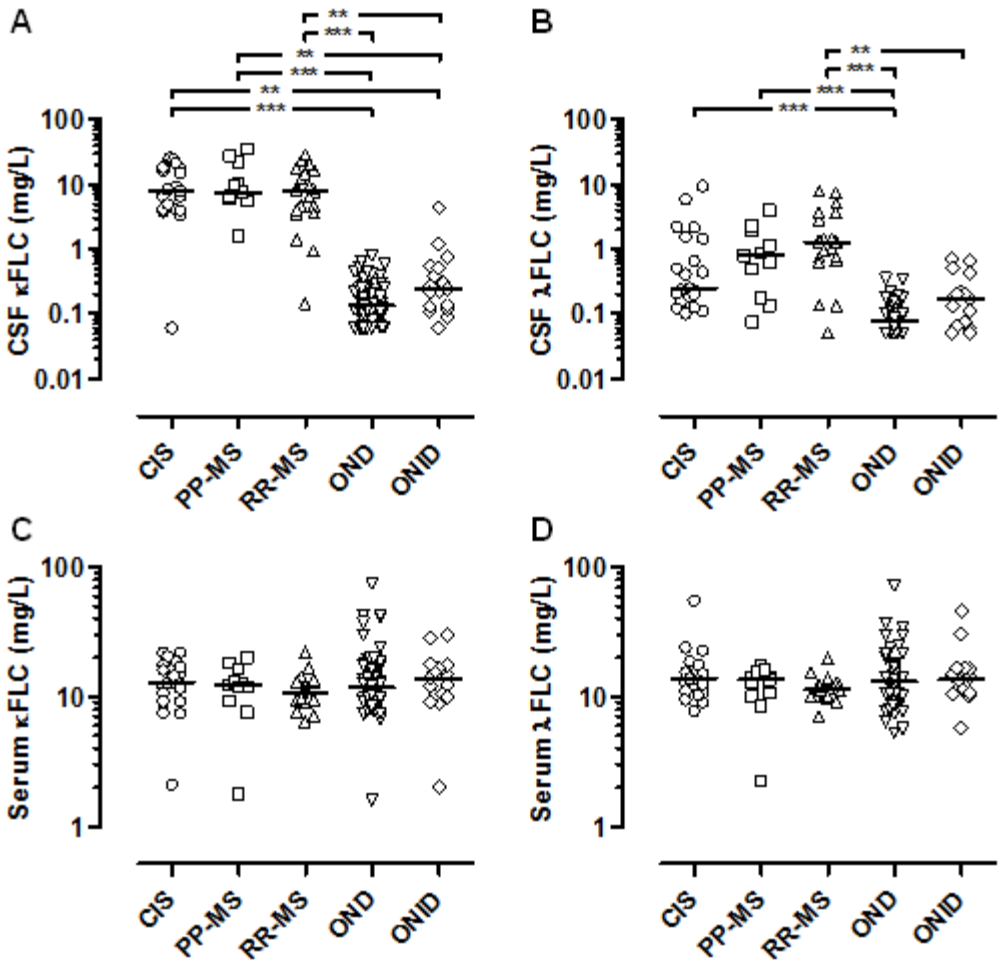

Fig. 1

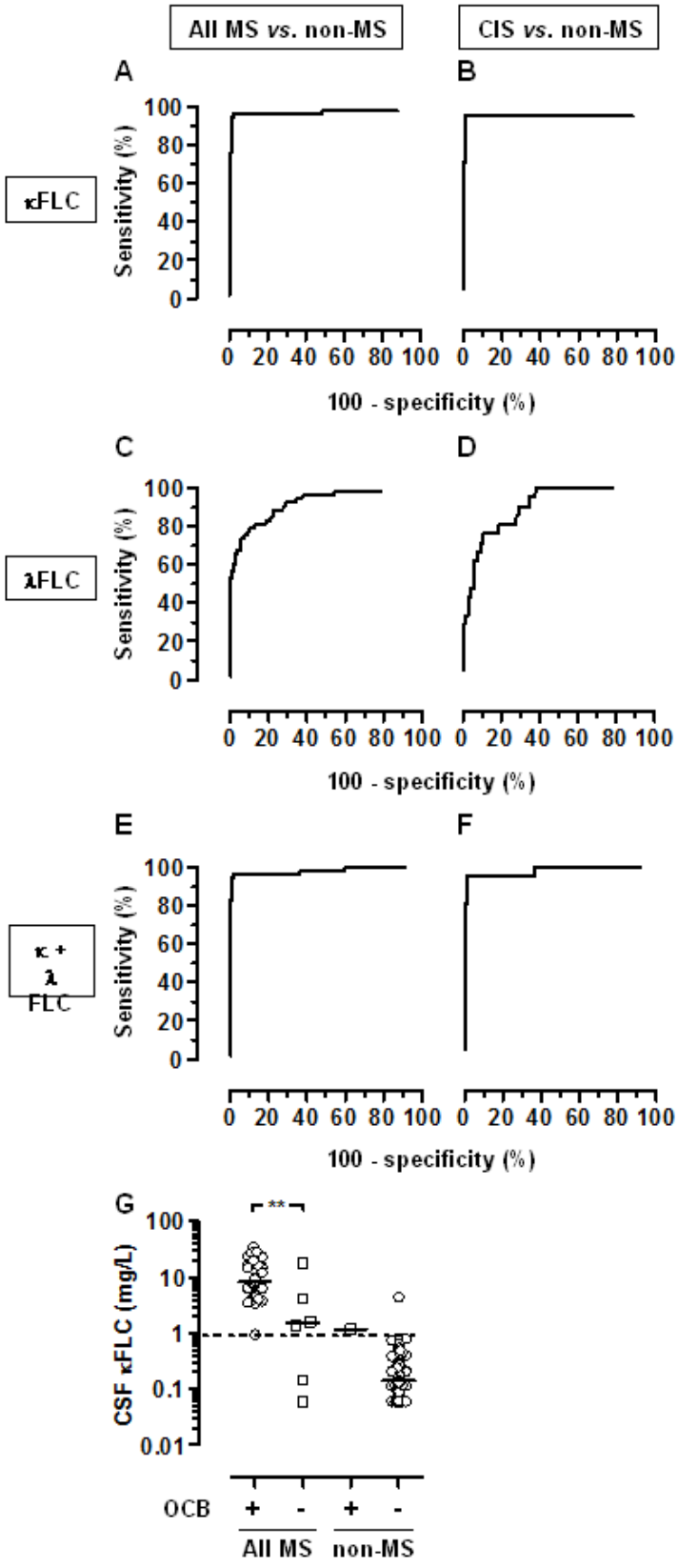

Fig. 2

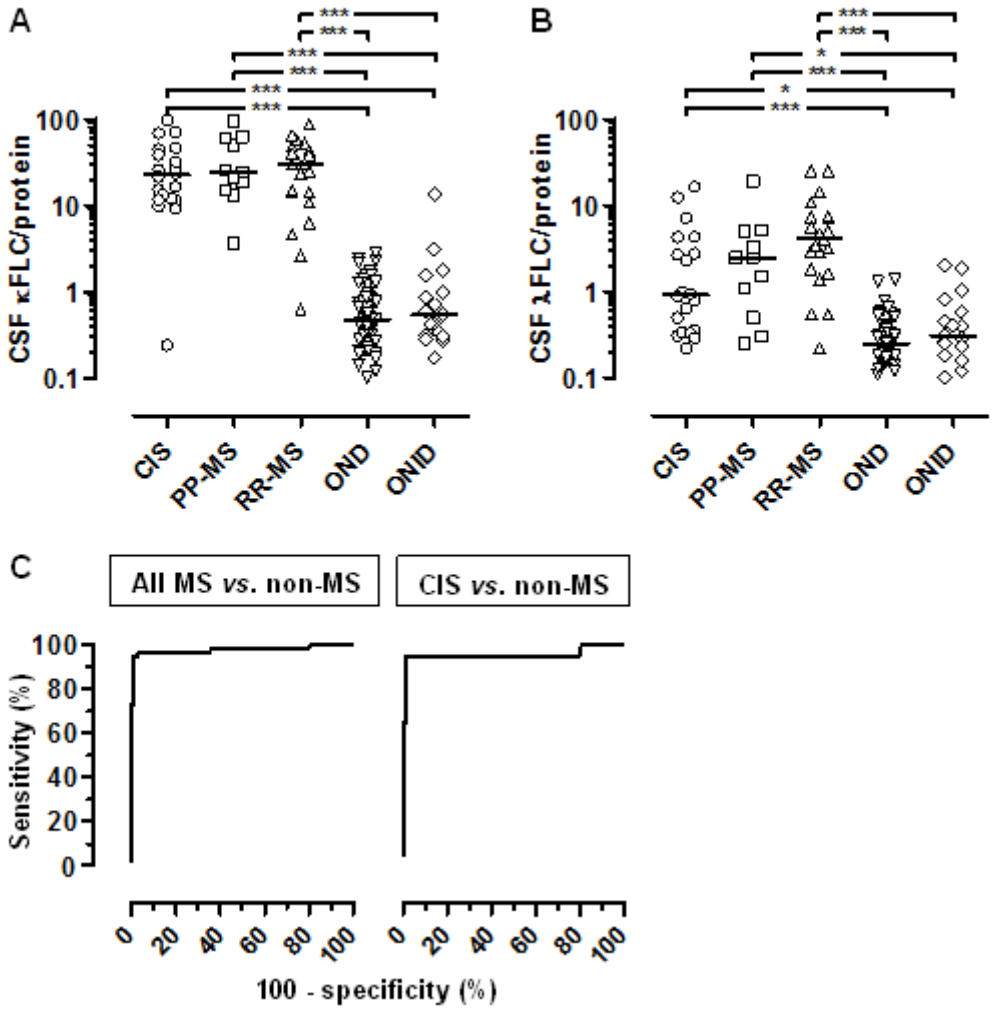

Fig. 3

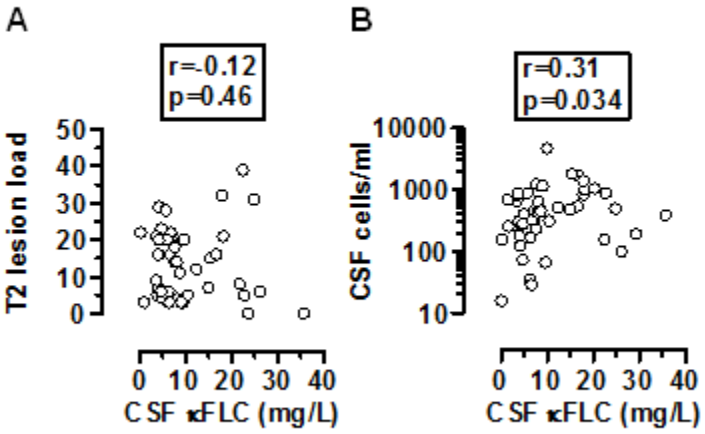

Fig. 4