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A multicenter study on the diagnostic significance of a single cerebrospinal fluid IgG band --Manuscript Draft--

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Corresponding Author:	Diana Ferraro University of Modena and Reggio Emilia ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Modena and Reggio Emilia
Corresponding Author's Secondary Institution:	
Corresponding Author E-Mail:	diana.ferraro@unimore.it
First Author:	Diana Ferraro
First Author Secondary Information:	
Order of Authors:	Diana Ferraro Diego Franciotta Roberta Bedin Claudio Solaro Eleonora Cocco Mario Santangelo Paolo Immovilli Alberto Gajofatto Massimiliano Calabrese Massimiliano Di Filippo Riccardo Orlandi Anna Maria Simone Francesca Vitetta Elisabetta Capello Debora Giunti Alessandra Murialdo Jessica Frau Sara Mariotto Antongiulio Gallina Claudio Gasperini Patrizia Sola
Order of Authors Secondary Information:	

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Abstract:	<p>The analysis of cerebrospinal fluid (CSF) and serum with isoelectric focusing can be of aid in the differential diagnosis of central nervous system (CNS) disorders. However, the diagnostic meaning of a single CSF-restricted IgG band is still uncertain. Aim of this multicenter study was to establish the frequency and disease associations of this finding.</p> <p>Of 9422 CSF reports, 151 (1.6%) showed a single CSF-restricted IgG band. Of the 129 patients with a definite diagnosis, 58.2% had CNS inflammatory-demyelinating diseases (the most frequent being Multiple Sclerosis: 21.7%), 6.2% tumours, 5.4% inflammatory peripheral nervous system diseases, and 30.2% miscellaneous diseases. At repeat CSF analysis, 3 out of 10 patients had developed an oligoclonal band pattern.</p> <p>Our results indicate that single CSF IgG bands tend to associate with diseases characterized by an intrathecal humoral immune response. This abnormality should alert clinicians of possible inflammatory disorders of the CNS and should, therefore, be reported.</p>
Suggested Reviewers:	<p>Francesco Lolli francesco.lolli@unifi.it Author of previous article on the same topic</p> <p>Gerard Davies gerard.davies@nhs.net Author of only other study on the follow-up of patients with a single CSF IgG band</p> <p>Hayrettin Tumani hayrettin.tumani@uni-ulm.de Expertise in Neuroimmunology</p> <p>Olaf Stuve olaf.stuve@utsouthwestern.edu Expertise in Neuroimmunology</p>
Author Comments:	<p>Dear Sir</p> <p>we would like to submit our manuscript entitled "A multicenter study on the diagnostic significance of a single cerebrospinal fluid IgG band" for consideration by Journal of Neurology as an original article.</p> <p>The detection of oligoclonal IgG bands (OCB) in paired serum and cerebrospinal fluid (CSF) samples is a valid tool in the differential diagnosis of central nervous system (CNS) disorders. Very few studies, however, have addressed the diagnostic significance of a single cerebrospinal fluid IgG band at isoelectric focusing (IEF), which falls into a grey area between 'no bands' (normality) and 'two bands' (pathological production of oligoclonal IgG within the CNS), and may even not be mentioned in the CSF analysis report.</p> <p>In a multicenter study we were able to evaluate the frequency and disease associations of this finding, which was present in 1.6% of 9422 CSF analyses (151 cases). The majority of the associated diseases had an inflammatory component of the CNS (e.g. multiple sclerosis and demyelinating, autoimmune or paraneoplastic CNS diseases) and an intrathecal humoral response is, thus, deemed plausible in these cases.</p> <p>We therefore strongly recommend reporting this IEF abnormality, since it can be of aid to clinicians during the diagnostic process and it should alert them of the possible presence of inflammatory CNS diseases.</p> <p>We hope that you will find our work to be of interest to Journal of Neurology, in light also of the previously published article on the same topic [Franciotta D, Bergamaschi R, Amato MP, et al (2005) Clinical correlations of CSF single IgG bands. J Neurol 252:1274-1275. doi: 10.1007/s00415-005-0820-1]</p> <p>Yours sincerely, Diana Ferraro</p>

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A multicenter study on the diagnostic significance of a

single cerebrospinal fluid IgG band

Diana Ferraro^a, Diego Franciotta^b, Roberta Bedin^a, Claudio Solaro^c, Eleonora Cocco^d, Mario Santangelo^e, Paolo Immovilli^f, Alberto Gajofatto^g, Massimiliano Calabrese^g, Massimiliano Di Filippo^h, Riccardo Orlandi^g, Anna Maria Simone^a, Francesca Vitetta^a, Elisabetta Capelloⁱ, Debora Giuntiⁱ, Alessandra Murialdo^j, Jessica Frau^d, Sara Mariotto^g, Antongiulio Gallina^h, Claudio Gasperini^k, Patrizia Sola^a and on behalf of the RIREMS group (Rising Italian Researchers in Multiple Sclerosis)

^a Neurology Unit, Department of Neurosciences, Nuovo Ospedale Civile S. Agostino Estense, University of Modena and Reggio Emilia, Via P. Giardini, 1355, Modena, Italy

^b Laboratory of Neuroimmunology, IRCCS, National Neurological Institute C. Mondino Foundation, Via Mondino 2, Pavia, Italy

^c Neurology Unit, Department of Head and Neck, ASL3 genovese, L.go Rosso, 2, Genova, Italy

^d Department of Medical Sciences and Public Healths, Cittadella di Monserrato, University of Cagliari, Cagliari, Italy

^e Neurology Unit, Ospedale Ramazzini, Via G. Molinari, 2, Carpi, Modena, Italy

^f Neurology Unit, Department of Specialistic Medicine, G. da Saliceto Hospital, Via T. Giuseppe, 49, Piacenza, Italy

^g Department of Neuroscience, Biomedicine and Movement, University Hospital of Verona, P.le Scuro 10, Verona, Italy

^h Neurology Unit, Medicine Department, University of Perugia, P.za Menghini, 1, Perugia, Italy

ⁱ Department of Neuroscience, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health, University of Genova and IRCCS AOU San Martino-IST, Largo R. Benzi, Genoa, Italy

^j Unit of Neurology, IRCCS San Martino University Hospital IST, Largo R. Benzi, Genoa, Italy.

^k Neurology Unit, Ospedale San Camillo Forlanini, Circonvallazione Gianicolense, 87, Rome, Italy

Corresponding author:

Diana Ferraro

Institution: Neurology Unit, Department of Biomedical, Metabolic and Neurosciences, University of Modena and Reggio Emilia, Modena, Italy

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Address: Nuovo Ospedale Civile Sant'Agostino Estense

Via Pietro Giardini, 1355

41126 Modena, Italy

Tel: +390593961722

Fax: +390593961323

E-mail: diana.ferraro@unimore.it

ORCID ID: 0000 0003 4818 3806

Key-words: cerebrospinal fluid; isoelectric focusing; single IgG band; Multiple Sclerosis

1. Introduction

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3 The detection of oligoclonal IgG bands (OCB) in paired serum and cerebrospinal fluid (CSF) samples is still considered
4 a valid diagnostic tool in the differential diagnosis of inflammatory diseases of the central nervous system (CNS),
5 notwithstanding the OCB ‘downgrading’ by the latest revision of the diagnostic criteria for multiple sclerosis (MS)
6 (reviewed in [1]). Two important consensus papers defined the most common OCB patterns that help clinical
7 pathologists and neurologists to interpret the results of serum and CSF analysis with the recommended isoelectric
8 focusing (IEF) techniques [2, 3]. However, additional IEF patterns, which have not been addressed by the leading
9 consensus papers [2, 3], can be detected in routine diagnostics [1]. In particular, the finding of a single IgG band in the
10 CSF falls into a grey area between ‘no bands’ (normality) and ‘two bands’ (pathological production of oligoclonal IgG
11 within the CNS). A few studies explored this grey area, and found that single CSF IgG bands associated with various
12 diseases including CNS infections, lymphomas, demyelinating/autoimmune/paraneoplastic diseases, peripheral nervous
13 system (PNS) diseases, and, above all, MS, with a highly variable frequency (from 0 to 44 %) [4–10]. Notably, these
14 studies are not fully comparable, due to the use of agarose electrophoresis, on which a single band corresponds to an
15 underlying single plasma cell clone, or of IEF, on which a monoclonal gammopathy yields 2-10 bands because of the
16 technique’s high resolving power that allows the detection of IgG microheterogeneity [1–3]. In addition to this
17 methodological heterogeneity, since only a small number of case series have been studied so far, the frequency and
18 diagnostic meaning of single CSF IgG bands is still an open question [1].

19
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21 In our multicenter retrospective study on single CSF IgG bands we aimed to assess: a) their frequency and disease
22 associations, b) possible differences in the demographic and other CSF features between the expectedly highly
23 represented cohort of MS patients and patients with other diseases, and c) the evolution over time of CSF IEF patterns
24 in a subgroup of patients who underwent follow-up spinal taps.

2. Patients and Methods

2.1 Patients

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Seven Italian neurological centres (Modena, Genoa, Cagliari, Carpi, Piacenza, Verona and Perugia) participated in this retrospective study. Patients showing a single CSF IgG band associated with identical bands in CSF and serum (‘mirror pattern’) (Figure, a), or alone (Figure, b) between January 2005 and June 2016 were selected from the centres’ databases. A single CSF IgG band within a mirror pattern was considered as if it was emerging from a polyclonal background, given that a mirror pattern is the product of a purely systemic oligoclonal B-cell activation, without

intrathecal IgG synthesis [2, 3]. All the CSF analyses were carried out for diagnostic purposes. The study was approved by the Modena Ethics Committee (protocol n. 188/15).

2.2 Demographic and CSF features

The following variables were collected: sex, age, date and rationale of CSF analysis (symptoms, diagnosis or suspected diagnosis), final confirmed diagnosis (if available), date of final confirmed diagnosis. CSF features included: CSF cell number and type, concentration of CSF total proteins, CSF/serum glucose ratio, IgG Index, CSF/serum albumin ratio, presence or absence of a 'mirror pattern' [2, 3].

2.3 CSF data sources and methods

Over 92% of the tested serum and CSF samples were analyzed in the three large laboratories of Modena (55%), Genoa (22%), and Cagliari (15%), with home-made agarose gel-based IEF techniques (Agarose IEF, Pharmalyte pH 3-10 and Pharmalyte pH 8-10.5 GE Healthcare), and a very similar protocol. Briefly, electrode strips were soaked with 0.05 or 1M sodium hydroxide (catholyte), and 0.05 or 1M sulphuric acid (anolyte). After focusing, the proteins were transferred to polyvinylidene difluoride (Modena), or nitrocellulose (Genoa and Cagliari) membranes, which were incubated with polyclonal rabbit anti-human IgG (Modena: Dako) or goat anti-human IgG (Genoa: DiaSorin; Cagliari: Sigma-Pierce), and then with alkaline phosphatase- (Modena) or peroxidase- (Genoa and Cagliari) conjugated polyclonal swine anti-rabbit (Modena: Dako) or rabbit anti-goat (Genoa and Cagliari: Dako) IgG. Membranes were finally stained with 5-Bromo-4-chloro-3-indolyl-phosphate and Nitro blue tetrazolium (Modena: Sigma-Aldrich), or with 3-Amino-9-ethylcarbazole (Genoa and Cagliari: Sigma-Aldrich). Focusing was performed on a Multiphor II apparatus (GE Healthcare) in all centres. All the other samples were analyzed in the laboratories of Verona (which used IsoGel Agarose IEF pH 3-10 Lonza, 1M sodium hydroxide and 0.5M carboxylic acid, polyclonal rabbit anti-human IgG-Dako, biotinylated anti-rabbit IgG-Vectastain ABC kit and 3-Amino-9-ethylcarbazole) and Perugia (which used Hydrigel 9 CSF isofocusing kit-Sebia).

Since 2000 the laboratories have been participating in external quality control schemes, annually organized by the Italian Association of Neuroimmunology (AINI). These schemes show that the analytical quality of OCB detection and the between-center agreement on the results of the external quality controls have substantially improved over the years (D. Fr., personal communication). As a proof of principle, the Laboratory of Neuroimmunology of Pavia, which is part of the AINI network, received 15 pairs of serum and CSF samples, with or without a single CSF IgG band, from the center of Modena, and blindly tested them, with a resulting strength of agreement that is considered to be 'fair' ($k=0.25$).

1 This result is in line, if not better than expected, with the fact that the IEF technique has low inter-laboratory
2 reproducibility, mainly due to the non-linearity of the pH gradient, and to the so called ‘cathodic drift’ [1] and that
3 ‘critical’ CSF samples, e.g., those with single CSF IgG bands, are at a high risk of misinterpretation (reviewed in [1]).
4

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6 As for the IEF track interpretation, the centers of Genoa and Modena exchanged and blindly assessed pictures of paired
7 CSF and serum samples of 112 patients. A single IgG band was concordantly identified in 91% of cases (kappa of
8 agreement, 0.82). Furthermore, the center of Modena re-tested 15 CSF and serum samples showing a single CSF IgG
9 band, with completely matching results.
10

11 2.4 Statistical methods

12 Absolute frequencies and percentages were calculated for categorical variables and the mean (\pm standard deviation)
13 and/or the median (\pm interquartile range) for continuous variables. Comparisons between groups were made using the
14 Kruskal-Wallis test for continuous variables and Fisher’s exact test for categorical variables, followed by post-hoc tests.
15

16 3. Results

17 3.1 Diagnoses

18 Out of a total of 9422 CSF analysis reports, 151 showed a single CSF-restricted IgG band (1.6%). A definite diagnosis
19 was available in 129/151 patients (Table 1). MS was the single disease with the highest frequency of single IgG band
20 detection. Cumulatively, MS and CNS inflammatory-demyelinating diseases (clinically isolated syndrome,
21 radiologically isolated syndrome and neuromyelitis optica spectrum disorder) accounted for 46.5% of all the cases
22 (Table 1).
23

24 3.2 Intra- and between-group comparisons

25 Twenty-eight of the patients with a single CSF IgG band were diagnosed with MS, 32 with CNS inflammatory-
26 demyelinating diseases and 69 with miscellaneous diseases. Mean age in patients with MS and in patients with CNS
27 inflammatory-demyelinating diseases was significantly lower than in patients with all the other diseases. Patients with
28 MS or with CNS inflammatory-demyelinating diseases had a lower frequency of single CSF IgG bands associated with
29 a mirror pattern in comparison with patients with miscellaneous diseases (Table 2)..
30

31 3.3 Follow-up findings

32 Ten patients underwent a second spinal tap after a mean interval of 458 days (range: 22-1482) from the first one. Three
33 patients (one with MS, one with progressive multifocal leukoencephalopathy and one with cerebral lymphoma)
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1 developed an oligoclonal pattern, while four patients (one with a compressive myelopathy, one with neurosarcoidosis,
2 one with motor neuron disease and one with anti- N-methyl D-aspartate receptor antibody encephalitis - following
3 treatment and remission) lost the single CSF IgG band, which remained unchanged in three patients with myelitis, viral
4 encephalitis or neuro- systemic lupus erythematosus.
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7 8 **4. Discussion** 9

10
11 Our main finding indicates that single CSF IgG bands preferentially associate with inflammatory diseases of the CNS.
12
13 Indeed, nearly one half of the cases were found in patients with MS or inflammatory-demyelinating CNS diseases in,
14 and another 12% in patients with CNS infections or autoimmune/paraneoplastic neurological diseases. A clear-cut and
15 mature CNS-restricted oligoclonal B-cell response is expected in all these disorders (reviewed in [1]). It is thus likely
16 that single CSF IgG bands might be the ‘tip of the iceberg’ of oligoclonal responses that are either mounting, or weak in
17 nature, if lumbar puncture is performed near to the onset of the disorder. The abnormality could, therefore, indicate a
18 ‘possible’ intrathecal IgG synthesis [6].
19
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22 As for the mean younger age found in patients with MS or CNS inflammatory-demyelinating diseases versus all other
23 patients, this finding likely depends on the typically younger age of MS onset. Analogously, the association between
24 single CSF IgG bands and a mirror pattern was more frequent in non-MS pathologies, probably because in the latter
25 conditions, including infectious disorders, a mirror pattern, which reflects a systemic B-cell activation, can be relatively
26 common [1].
27
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29 Table 3 summarizes the main findings of other studies exploring the diagnostic significance of a single IgG band.
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33 Cumulatively, these findings confirm our data on the preferential association between single CSF IgG bands and CNS
34 inflammatory disorders. The plausibility of such an association is reinforced by the fact that 3 of 10 of our patients with
35 repeated lumbar punctures developed OCB (one case of conversion from Clinically Isolated Syndrome to MS), which is
36 in line with the data of the only other follow-up study on the topic [5]. The study showed that 33% of the patients with a
37 single CSF IgG band later manifested CSF OCB (two thirds of them had inflammatory demyelinating diseases),
38 whereas in approximately 50% of them the pattern remained unchanged [5].
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42 The transition from single CSF IgG band patterns to full-blown OCB patterns in MS is not surprising, as many studies
43 have shown intra-patient variations of OCB patterns in this disease over time [11–13], with a high number of intrathecal
44 B-cell clones in patients with the longest disease duration [12] .
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48 In very rare cases, single CSF bands can be expected in cerebral lymphomas [5, 8, 14]. In the present series too, there is
49 a patient with a cerebral marginal zone lymphoma. Four additional patients had hematopoietic malignancies
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(lymphomas in 3 and chronic lymphocytic leukemia in one patient). Although reported in one case [15], single CSF bands in such systemic disorders seem to be incidental rather than disease-related.

On the other hand, the present and the previous studies [4, 5, 7] show that in about one third (17-44%) of patients with single CSF IgG bands, the associated diseases are either vascular or degenerative in nature, or they affect the PNS. An intrathecal IgG production is, therefore, unexpected.

In these cases, it is likely that finding a single CSF IgG band might derive from misinterpretations of the IEF tracks in a few cases at least, due to the artifactual banding that is typical of IEF, and as suggested by the results of the AINI external quality control schemes [1]. In the IEF-OCB field, some degree of inter-laboratory variability is more than expected (but no study has ever addressed this issue), as IEF is a technique that is inherently quite impossible to standardize. This shortcoming is an objective and insoluble limit of any multicenter study on the topic, including the present one. Both the inclusion of single CSF IgG band false positive cases, and the exclusion of false negative ones can thus have affected the study findings. However, the fact that the results did not differ substantially from those of the previously published studies [4–10], suggests that the rate of incorrect IEF track interpretation might be acceptable.

Moreover, the three laboratories that examined more than 90% of paired serum and CSF samples participate in the AINI external quality control schemes, and some procedures aimed at boosting the reliability of the study have been performed.

As for all the other diseases, 5% of single CSF IgG bands were detected in patients with inflammatory PNS diseases, in line, again, with the other studies on the topic (Table 3). In this case, it is difficult to ascribe the abnormality to a weak or emerging intrathecal IgG production, as in the case of MS. Indeed, data that favour the hypothesis of a CNS-restricted intrathecal B-cell activation to intrathecal portions of peripheral nerves [5], or to PNS-CNS shared autoantigens [16] have not yet been substantiated by the evidence of full-blown CSF OCB. The very high frequency of a CSF-restricted monoclonal IgG band reported by Dalakas and Colleagues in 14/15 patients with chronic relapsing polyneuropathy [17] has never been confirmed.

CSF OCB have been described with very low frequencies in neurovascular [10, 18, 19] and in neurodegenerative [10, 20] diseases, but, as in the case of patients with headache or psychiatric disorders, single CSF IgG bands are more likely traces of previous contacts between infectious agents and the immune system (immunologic memory) than abnormalities related to ongoing intrathecal B-cell activation.

5. Conclusion

Our data show that the single most frequent diagnosis in patients with a single CSF-restricted IgG band is MS, followed by diagnoses of inflammatory disorders of the CNS. Overall, these findings indicate that single CSF IgG bands tend to

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2 associate with diseases characterized by an involvement of intrathecal humoral immune responses, and strongly support
3 the notion that this abnormality should alert clinical pathologists and neurologists of a possible presence of
4 inflammatory disorders of the CNS. Given such an important diagnostic weight, although rare, single CSF IgG bands
5 should always be reported, and in uncertain clinical cases, the lumbar puncture should be repeated after 6-12 months.
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8 **Figure Legend**

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11 **Figure** Examples of isoelectric focusing patterns characterized by a single CSF IgG band associated with identical
12 bands in CSF and serum ('mirror pattern') (a), or alone (b)
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15 CSF: cerebrospinal fluid; S: serum; \oplus : anode; \ominus : cathode
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25

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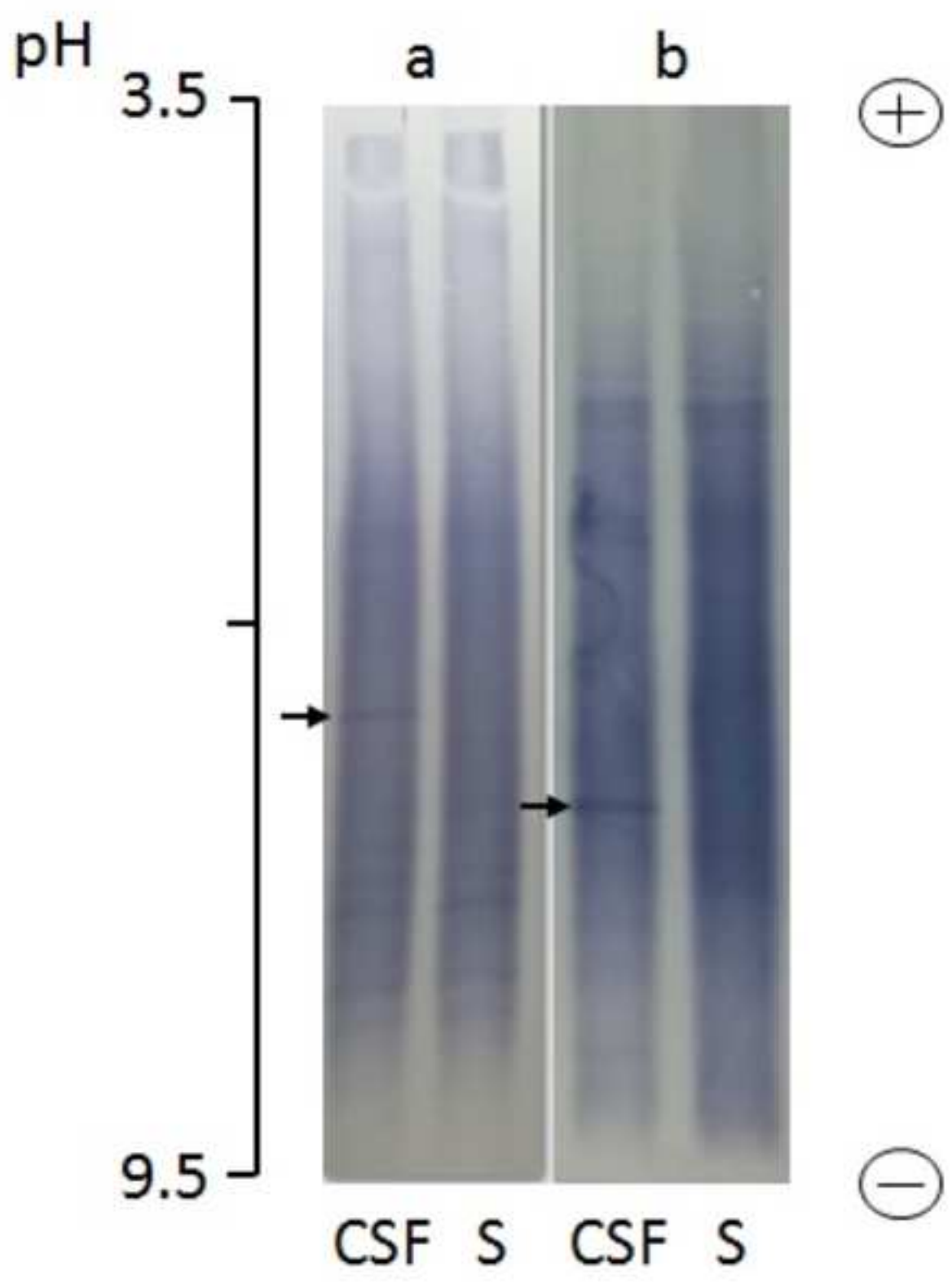


Table 1. Diagnoses associated with a single CSF IgG band in our cohort of 129 patients

Diagnosis	Number of patients (%)
Multiple Sclerosis	28 (21.7%)
CNS inflammatory-demyelinating diseases ¹	32 (24.8%)
CNS infections ²	9 (7%)
Tumours ³	8 (6.2%)
Inflammatory PNS diseases ⁴	7 (5.4%)
CNS autoimmune/paraneoplastic neurological diseases ⁵	6 (4.7%)
Miscellaneous diagnoses ⁶	39 (30.2%)

¹clinically isolated syndrome, radiologically isolated syndrome and neuromyelitis optica spectrum disorder ;

²*Borrelia burgdorferi*, Herpes simplex virus, John Cunningham virus, *treponema pallidum*; ³tumours within/adjacent to the CNS: cerebral marginal zone lymphoma, cerebral glioma, dysembryoplastic neuroepithelial tumour, vertebral metastasis; hematologic tumours: chronic lymphocytic leukemia, Non-Hodgkin lymphoma, hepatic-pulmonary lymphoma; ⁴Guillain-Barrè syndrome, anti-MAG neuropathy, multifocal motor neuropathy, chronic inflammatory demyelinating polyneuropathy; ⁵neuro-systemic lupus erithematosus, Bickerstaff encephalitis, anti-phospholipid syndrome, cerebral vasculitis, anti- N-methyl D-aspartate receptor antibody encephalitis; ⁶idiopathic intracranial hypertension, spondilomyelopathy, cerebral ischaemia, vascular encephalopathy, amyotrophic lateral sclerosis, polyneuropathy, Alzheimer's disease, fronto-temporal dementia, neurosarcoidosis, Tolosa-Hunt syndrome, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, Melkersson-Rosenthal syndrome, reversible cerebral vasoconstriction syndrome, HCV-related multineuropathy, DYT1-positive dystonia, cerebral venous thrombosis, migraine, tension-type headache, syndrome of transient headache and neurologic deficits with cerebrospinal fluid lymphocytosis, spinal dural fistula, psychiatric conditions (catatonia, conversion disorder), post-traumatic epilepsy, perinatal paraparesis, transient global amnesia

Table 2 Demographic and cerebrospinal fluid characteristics of patients with a single cerebrospinal fluid IgG band with multiple sclerosis, CNS inflammatory-demyelinating and miscellaneous diseases

	Multiple sclerosis (n=28)	CNS inflammatory-demyelinating diseases (n=32)	Miscellaneous diseases (n=69)	p-value
Age (years) (mean \pm SD)	37.7 \pm 13	37.2 \pm 15	50.3 \pm 19	*0.003
Sex (Male/Female)	13/15	10/22	27/42	ns
CSF cell count (number/ μ l), median (IQR)	3 (1-7)	1.5 (1-3.5)	1 (1-2)	ns
CSF total protein (g/dl), median (IQR)	47 (27-59)	37 (33-49)	37 (30-48)	ns
IgG index, median (IQR)	0.53 (0.5-0.65)	0.56 (0.5-0.6)	0.58 (0.5-0.66)	ns
CSF/serum albumin, median (IQR)	5 (3.1-6.7)	4.7 (3.6-6.3)	4.6 (3.7-6.3)	ns
Additional mirror pattern (yes/no)	2/26	3/29	17/49	∞ 0.04

SD= standard deviation; IQR= interquartile range

*post-hoc tests: MS versus CNS inflammatory-demyelinating diseases: $p=0.45$; CNS inflammatory-demyelinating diseases versus miscellaneous diseases: $p=0.0003$; MS versus miscellaneous diseases: $p=0.001$

∞ post-hoc tests: MS versus CNS inflammatory-demyelinating diseases: $p=0.6$; CNS inflammatory-demyelinating diseases versus miscellaneous diseases: $p=0.048$; MS versus miscellaneous diseases: $p=0.032$

Table 3. Studies exploring the frequency and diagnostic meaning of single cerebrospinal fluid-restricted IgG bands

Authors	N/total (%)	MS N (%)	CNS inflammatory / autoimmune/ paraneoplastic disease N (%)	PNS inflammatory disease N (%)	CNS infection N (%)	CNS tumor N (%)	Seemingly unrelated disease N (%)
McCombe et al [8]	3/1490 (0.2%)	-	1 (33)	1 (33)	-	1 (33) (lymphoma)	-
Ben-Hur et al [4]	33/6000 (0.6%) (20 with diagnosis)	7 (35)	9 (45)	3 (15) (1 had MS too)	-	-	2 (10) ¹
Franciotta et al [7]	61/3273 (1.9%)	27 (44)	8 (13)	1 (2)	16 (26)	-	9 (15) ²
Davies et al [5]	31 (27 with diagnosis)	3 (11)	7 (26)	-	4 (15)	1 (4) (lymphoma)	12 (44) ³

N= number; ¹ torsion dystonia, superficial hemosiderosis of the CNS; ²polyneuropathy (n=5), Alzheimer's disease (n=2), retrobulbar optic neuropathy (n=2); ³axonal neuropathy (n=2), vascular disease (n=2), other/no diagnosis (n=8): orthostatic tremor, migraine+/-, oscillopsia, intermittent sensory symptoms, cerebral venous infarction, progressive cerebellar syndrome, chronic myalgia