Esther Ganelin-Cohen*, Sizilia Golderman, Regina Yeskaraev, Ayal Rozenberg, Avi Livneh and Batia Kaplan

Search for new biomarkers of pediatric multiple sclerosis: application of immunoglobulin free light chain analysis

https://doi.org/10.1515/cclm-2017-0911
Received October 9, 2017; accepted December 18, 2017; previously published online February 6, 2018

Abstract

Background: Identifying new biomarkers is needed to overcome the diagnostic difficulties of pediatric multiple sclerosis (MS). Recently, we developed a new technique including CSF analysis of free light chain (FLC) monomers and dimers, which can improve diagnosis of adult MS. The present study has been designed to evaluate the utility of our technique for MS diagnosis in children.
Methods: Patients with MS (n = 21) and non-MS demyelinating or inflammatory neurological disorders (n = 35) participated in the study. MS diagnosis was based on clinical and magnetic resonance imaging (MRI) findings. Western blot analysis was applied to examine FLC in the patients' CSF and serum. FLC indices for FLC monomer and dimer levels and κ/λ ratios were estimated. The samples were also analyzed by oligoclonality test.
Results: The study revealed abnormally elevated levels of κ-FLC monomers and dimers in the CSF of 10 MS patients ("κ-type MS"). Increased amounts of λ dimers were found in six MS cases ("λ-type MS"), while high levels of both κ and λ FLC ("mixed type MS") were documented in three MS cases. MRI and clinical assessment showed a more aggressive disease form for the "mixed" and "λ-type" cases. Our method demonstrated higher sensitivity (90.5%) and specificity (91.4%) for discrimination between MS and non-MS patients, as compared to oligoclonality test (81% and 65.7%, respectively).
Conclusions: The proposed method may significantly contribute to diagnosis and prognosis of pediatric MS.

Keywords: cerebrospinal fluid; dimers; free light chains; monomers; pediatric multiple sclerosis.

Introduction

Pediatric multiple sclerosis (MS) is considered a rare disease, accounting for only 3%–5% of all MS cases [1]. The first symptoms of MS often appear as individual demyelinative events, which can be clinically expressed as optic neuritis (ON), transverse myelitis (TMY) or show other neurological signs due to brainstem, cerebellar or hemispheric dysfunction. A growing body of evidence suggests that early treatment may slow the progression of MS [2]. Prediction and characterization of the future attacks might be helpful, but, unfortunately, such prognostic tests are still missing.

MS diagnosis is based on the combination of a typical demyelinative clinical presentation, which is not specific but suggestive for MS, plus a typical dynamic magnetic resonance imaging (MRI), which fits the McDonald criteria [3]. However, despite high sensitivity of MRI, other central nervous system (CNS) diseases may mimic MS radiologically [4]. In children the differential diagnosis is wider as compared to adults, even within the demyelinating spectrum disorder *per se* (e.g. acute disseminated encephalomyelitis [ADEM] vs. MS) [5]. In such cases a proper interpretation of the MRI findings might be difficult, and supporting laboratory data may be needed for correct diagnosis. In the commonly used diagnostic laboratory test, the demonstration of oligoclonal immunoglobulin (Ig) bands indicates an intrathecal production of Igs and supports the diagnosis of MS. Yet, the sensitivity of this method in pediatric MS is not clear, as it varies significantly in different studies [6]. Thus, a search for new biomarkers of pediatric MS, and developing of new, more

***Corresponding author: Esther Ganelin-Cohen,** MD, PhD, Institute of Pediatric Neurology, Schneider Children's Medical Center, Petach Tikva 49202, Israel, E-mail: esterg2@clalit.org.il; and Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel
Sizilia Golderman and Batia Kaplan: Heller Institute of Medical Research, Sheba Medical Center, Tel-Hashomer, Ramat Gan, Israel
Regina Yeskaraev: Department of Clinical Biochemistry, Sheba Medical Center, Tel-Hashomer, Ramat Gan, Israel
Ayal Rozenberg: Department of Neuroimmunology, Rambam Health Care Campus, Haifa, Israel
Avi Livneh: Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel; and Heller Institute of Medical Research, Sheba Medical Center, Tel-Hashomer, Ramat Gan, Israel

precise diagnostic techniques is crucial for effective and timely treatment.

Intrathecal production of κ and λ Ig free light chains (FLC) is now regarded as an important immunological response developing in the CNS of MS patients. Thanks to the development of highly sensitive nephelometric FLC assays, the importance of FLC analysis in MS diagnosis was established. The nephelometric assay, allowing the quantification of total level of monomeric plus dimeric FLC, showed a significant increase of κ-FLC in the CSF of MS patients [7–9]. Yet, the specificity of this assay for the diagnosis of MS remained lower than that of the oligoclonality test [9] and even of the IgG index [8].

Recently, we employed a semiquantitative Western blotting technique to analyze FLC in the CSF-serum sample pairs of adult patients with MS and related diseases [10–12]. In contrast to the previously used FLC assays [7–9], we differentially analyzed the monomeric and covalently-bound dimeric forms of FLC. A number of the diagnostically useful FLC indices were established by us to distinguish MS from other neurological diseases in adults. The specificity and sensitivity of our method were both high compared to the oligoclonality test.

In the present study, aimed to reveal new reliable biomarkers of the pediatric MS, we applied our new methodological approach of FLC analysis and showed the utility of this technique in the MS diagnosis in children.

Materials and methods

Patients and samples

CSF and serum samples, collected from 64 pediatric patients (age range 1–17 years), were stored at –20 °C temperature until used. The patient groups were defined as follows:

1. Patients with definite MS diagnosis (n = 21);
2. Patients with other demyelinating neurological disorders (n = 20), including (ADEM, n = 3), neuromyelitis optica (NMO, n = 2), clinically isolated syndrome (CIS, n = 8), radiologically isolated syndrome (RIS, n = 2), transverse myelitis (TMY, n = 2), optic neuritis (ON) with the seropositivity to myelin oligodendrocyte glycoprotein (MOG+, n = 1), Guillain-Barré syndrome (GBS, n = 2);
3. Patients with non-demyelinating neurological diseases (n = 15). These included cases with vasculitis (n = 1), Susac syndrome (n = 1), Rasmussen's encephalopathy (RE, n = 3, one of them electrical status epilepticus in sleep [ESES]), epilepsy (n = 2), Hashimoto disease (n = 1), migraine (n = 3), cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL, n = 1), Sydenham's chorea (n = 1), monoplegia of left upper extremity (n = 1), non-MS (patient under observation, n = 1);
4. Control group (negative control) of patients (n = 8) with no evidence for demyelinating, inflammatory, autoimmune, or other well-defined neurological disease.

MS diagnosis was based on dissemination in time and space as demonstrated by clinical and MRI findings [3]. The study was approved by the institutional review board (Rabin Medical Center, 0104-15-RMC). All participants have signed the written informed consent forms.

Oligoclonality test

A standard procedure for detection of IgG bands was used by employing high-resolution agarose gel electrophoresis (Hydragel six CSF) coupled with immunofixation (Sebia, Moulineaux, France) [10, 11].

FLC analysis by Western blotting

CSF and serum samples were obtained from each patient participating in this study. Preparation of CSF and serum samples for Western blot analysis was performed as described in [11] with some modifications. CSF sample was mixed with electrophoresis sample buffer (S.B.x5), 2:1 (vol/vol). Serum sample was diluted with phosphate buffered saline (PBS), 1:40 (vol/vol), and the diluted serum sample was mixed with S.B.x2, 1:3 (vol/vol). CSF and serum samples were run on 10%–20% Nu-Sep Tris-Tricine gels (Gradipore, Frenchs Forest, Australia) under non-reducing conditions and blotted onto nitrocellulose (Schleicher and Schuell, Dassel, Germany). Rabbit antibodies to human Ig κ and λ light chains (DAKO, Carpinteria, CA, USA) were used as primary antibodies. Peroxidase conjugated goat anti-rabbit IgG antibodies were used as secondary antibody (Sigma-Aldrich, Israel). Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) was used to visualize immunoreactive bands on X-ray films (FujiFilm Corp., Tokyo, Japan) at exposure times from 5 to 50 s.

Software [10] was used for quantitative evaluation of the intensity of FLC immunoreactive bands. The obtained intensity value in the tested CSF sample was normalized relative to that in the control sample: intensity of FLC band of positive control divided by intensity of FLC band of tested sample. The positive control sample, representing a mixture of CSF samples obtained from 15 patients with definite MS diagnosis, was included in each electrophoretic run alongside the tested samples. The following FLC indices (I) were calculated:

1. I_0 and I_1 – CSF λ and κ monomer level indices, respectively,
2. I_2 – CSF monomeric κ/λ ratio index,
3. I_3 – index to compare the κ/λ monomer ratio in the CSF vs. that in the serum ($\kappa/\lambda_{SERUM}/\kappa/\lambda_{CSF}$),
4. I_4 – CSF λ dimer level index,
5. I_5 – CSF λ dimerization index: λ dimer/monomer ratio (λD/M),
6. I_6 – index to compare the λ dimerization in the CSF to that in the serum ($\lambda D/M_{SERUM}/\lambda D/M_{CSF}$).

Of importance, I_0, I_1, I_2, I_4 and I_5 indices are calculated using normalized intensity values, while I_3 and I_6 are calculated without normalization. (Notably, the lower index values point to the higher FLC levels, higher κ/λ ratios and higher λ dimerization values.)

Statistical analysis

Fisher's exact two-tailed test was applied to compare the three distinct MS patient subgroups (characterized by the differences in their FLC monomer-dimer patterns; see Results) with respect to the clinical and MRI data.

Results

FLC patterns in the MS patients

Analysis of the CSF-serum samples from control individuals and MS patients revealed striking differences between these two groups in relation to their FLC monomer-dimer (M-D) patterns in the CSF. The control individuals (n = 8) demonstrated a weak or no immunoreactivity in the area corresponding to FLC monomers (25 kDa) and dimers (50 kDa) in the CSF. In contrast, 19 out of 21 MS patients showed significantly increased levels of κ-FLC monomers and dimers, and/or increased levels of λ dimers (Figure 1).

To establish the clear-cut FLC criteria allowing the differentiation of MS from other non-MS neurological diseases, we determined quantitatively the intensities of immunoreactive FLC bands which were then used to calculate the FLC indices (I) as already described. The calculated FLC indices of pediatric patients with the clinically definite MS diagnosis are displayed in Table 1. The 10 MS cases (patients #1–10) showed high intensity of immunoreactive κ-FLC bands (I_1 mean = 1.12 ± 0.96) together with the relatively low immunoreactivity of λ monomers (I_0 mean = 9.21 ± 8.71) and dimers (I_4 mean = 8.29 ± 7.87). Also, κ/λ ratio of monomeric FLC in the CSF was significantly higher than that in the serum of the same patient (I_3 mean = 0.19 ± 0.13) ("κ type" FLC pattern, Figure 1B). In six MS cases (patients #11–16), the major abnormality was manifested by high intensity of dimeric λ-FLC bands (I_4 mean = 0.85 ± 0.67); in these cases the value of λ dimerization was much higher in the CSF than that in the serum (I_6 mean = 0.09 ± 0.007). Of note, the κ monomer levels varied widely (I_1 from 0.67 to 18) among these five patients ("λ type" FLC pattern, Figure 1C). Three MS cases (patients #17–19) showed high levels of κ FLC (monomers and dimers) and λ dimers ("mixed type" FLC pattern, Figure 1D). In all these MS cases the immunoreactivity of λ monomer bands was relatively weak (I_0 mean = 9.21 ± 8.71).

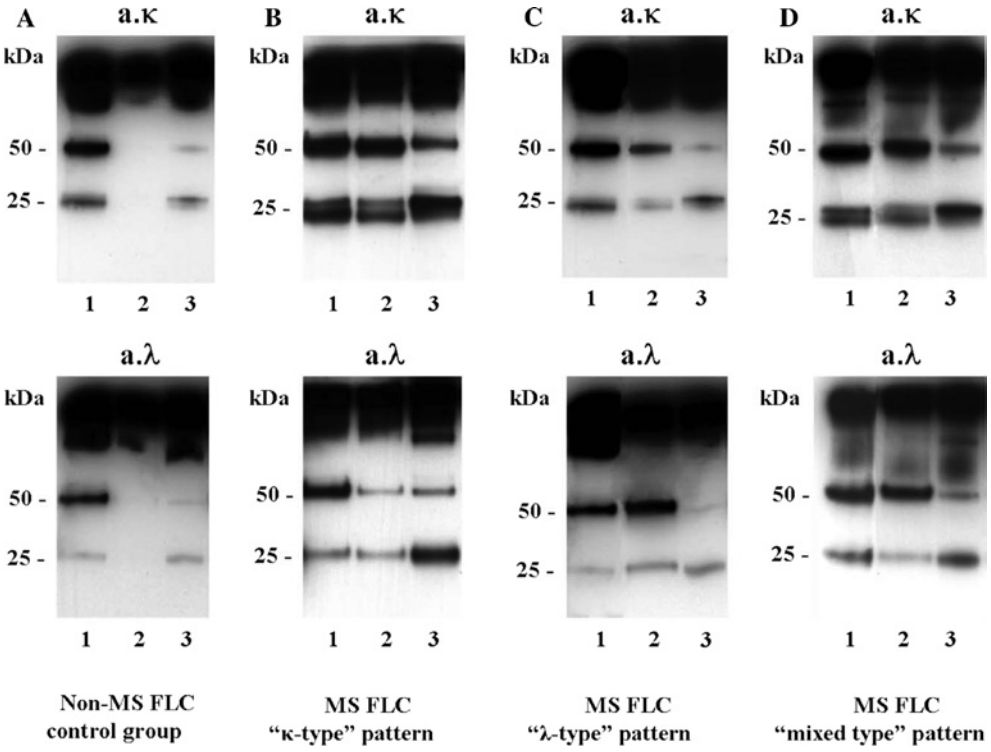

Figure 1: Western blot analysis of the FLC monomer-dimer patterns in MS patients (B, C, D) and control individual/negative control (A). FLC bands were detected using anti-κ (a.κ) and anti-λ (a.λ) antibodies. 1-CSF positive control (MS), 2 and 3 – the tested CSF and serum samples, respectively. (B–D) – MS patients with "κ-type", "λ-type" and "mixed type" FLC patterns, respectively.

Table 1: MS patients: data of FLC analysis and OCB test versus clinical diagnosis.

Patient #	Age, years	I_0	I_1	I_2	I_3	I_4	I_5	I_6	FLC test: MS/non-MS	OCB test	Clinical diagnosis
1	16	7.0	0.89	0.13	0.16	10.17	1.45	0.03	MS κ-type	+	MS
2	14.5	2.30	1.10	0.48	0.18	13.60	5.88	0.40	MS κ-type	Inconclusive	MS
3	16	15.0	0.41	0.03	0.09	6.33	0.42	0.29	MS κ-type	+	MS
4	17	1.60	0.84	0.53	0.36	1.02	0.64	0.14	MS κ-type	+	MS
5	16	0.35	0.19	0.55	0.40	1.26	3.57	0.23	MS κ-type	+	MS
6	16	22.0	1.67	0.08	0.02	18.22	0.83	0.01	MS κ-type	+	MS
7	17	7.75	1.75	0.23	0.10	12.00	0.67	1.3	MS κ-type	Inconclusive	MS
8	17	6.0	0.47	0.08	0.26	3.57	0.60	0.13	MS κ-type	+	MS
9	15	0.55	0.44	0.81	0.37	1.0	1.82	0.09	MS κ-type	+	MS
10	14.5	27.0	1.91	0.07	0.04	25.64	0.95	0.06	MS κ-type	+	MS
11	11.5	0.40	2.36	5.91	3.66	0.57	1.43	0.01	MS λ-type	+	MS
12	10.5	3.50	18.0	4.0	1.20	2.06	0.59	0.03	MS λ-type	+	MS
13	15	0.40	2.58	6.45	2.17	0.57	1.41	0.12	MS λ-type	+	MS
14	15	0.23	0.67	2.91	1.84	0.42	1.82	0.19	MS λ-type	+	MS
15	16	0.7	1.7	2.53	3.01	0.72	1.08	0.094	MS λ-type	+	MS
16	16	15.00	3.53	0.47	0.18	1.54	0.11	0.04	MS λ-type	+	MS
17	14	9.5	0.51	0.03	0.02	2.58	0.27	0.04	MS mixed type	+	MS
18	14	2.25	0.52	0.23	0.07	3.0	1.33	0.04	MS mixed type	Inconclusive	MS
19	7	0.75	0.18	0.24	0.15	0.86	1.14	0.05	MS mixed type	+	MS
20	14.5	6.00	8.00	1.33	2.62	5.00	0.83	0.06	Non-MS	+	MS
21	11	1.33	n.m	n.m	n.m	19.33	14.29	0.18	Non-MS	–	MS

OCB test positive (+), presence of ≥2 bands in CSF, and their absence in serum; OCB test negative (–), absence of oligoclonal bands in CSF; OCB test is inconclusive, a very weak immunostaining intensity of bands.

Based on these calculations, the FLC criteria were established to support the diagnosis of pediatric MS (conditions A or B):

1. In case of MS (conditions A or B), $I_0 \geq 0.2$ and $I_1 < 20$.
2. A: $I_1 \leq 3$ and $I_2 \leq 1$ and $I_3 \leq 0.7$;
3. B: $I_4 \leq 0.8$ or $I_4 \leq 3$ and $I_5 \leq 2$ and $I_6 \leq 0.05$.

The obtained results were also helpful to determine three different pathological patterns of FLC observed in MS:

1. "κ type" FLC pattern: only conditions A are satisfied,
2. "λ type" FLC pattern: only conditions B are satisfied,
3. "Mixed type" FLC pattern: both conditions (A and B) are satisfied.

FLC patterns in the non-MS patients

In patients with non-MS neurological diseases, the FLC patterns of 31 out of 34 cases were different from those observed in MS (Table 2). In two out of seven CIS cases the immunoreactivity of FLC monomer and dimer bands in the CSF was very weak as compared to MS (patients #24 and #26). In other two CIS patients (#23 and #25) the κ-FLCs were not detectable, while the λ-FLC bands were very weak (Figure 2A, case 23, track 4). The CIS patient #27 showed only slight increase in FLC levels, which was incompatible with MS ($I_1 = 10.5$ and $I_4 = 6.5$). In one of seven CIS cases (patient #22), κ monomer level was significantly increased ($I_1 = 1.96$), but in contrast to MS, no abnormalities were found by comparing the κ/λ monomer ratio in the CSF versus that in the serum ($I_3 = 1.63$). It is remarkable, that this patient showed seropositivity to myelin basic protein (MBP). The CIS patient #28 showed FLC pattern typical of MS; of note, this patient converted to MS during the 1st year of the patient follow-up.

In ADEM, each of the three tested cases showed different FLC patterns, but none of the patterns resembled MS. The abnormally high levels of κ monomers were observed in two NMO cases: $I_1 = 0.71$ (patient #32) (Figure 2B, track 4), and $I_1 = 1.09$ (patient #33). However, the other FLC indices in these NMO patients showed no abnormalities typical of MS (Table 2). One patient with vasculitis (# 44, Susac syndrome) displayed the FLC pattern typical of MS. In the second vasculitis patient (#45), κ-FLCs were not measurable and only slight increase of λ dimer level was observed. Also, in one of the two RIS cases (patient #36) FLC pattern was close to normal, while the second RIS patient (#37) showed the MS-like FLC pattern (this patient had a family history of MS).

Table 2: Non-MS patients: data of FLC analysis and OCB test versus clinical diagnosis.

Patient #	Age, years	I_0	I_1	I_2	I_3	I_4	I_5	I_6	FLC test: MS/non-MS	OCB test	Clinical diagnosis
22	11.5	0.36	1.96	5.39	1.63	3.30	9.09	0.39	Non-MS	Inconclusive	CIS
23	13	6.00	n.m	n.m	n.m	12.92	2.17	0.31	Non-MS	–	CIS
24	14	n.m	n.m	n.m	n.m	n.m	n.m	n.m	Non-MS	–	CIS
25	8	3.20	n.m	n.m	n.m	14.40	4.55	0.15	Non-MS	–	CIS
26	15.5	n.m	n.m	n.m	n.m	n.m	n.m	n.m	Non-MS	–	CIS
27	14	3.10	10.50	3.30	1.14	6.50	2.00	0.17	Non-MS	–	CIS
28	16	3.40	2.14	0.63	2.25	1.29	0.40	0.01	MS λ	+	CIS
29	2.5	0.86	n.m	n.m	n.m	0.61	0.71	0.00	Non-MS	–	ADEM
30	3	7.50	n.m	n.m	n.m	3.66	n.m	n.m	Non-MS	+	ADEM
31	14	0.04	n.c	n.c	n.c	n.c	n.c	n.c	Non-MS	+	ADEM
32	11.5	0.20	0.71	3.57	4.84	10.00	50.00	1.39	Non-MS	–	NMO
33	17.5	1.00	1.09	1.09	1.90	2.71	2.70	0.10	Non-MS	+	NMO
34	4	n.m	n.m	n.m	n.m	6.33	n.m	n.m	Non-MS	–	TMY
35	5	4.00	6.60	1.60	0.70	18.66	4.76	0.37	Non-MS	–	TMY
36	16.5	1.00	n.m	n.m	n.m	7.50	n.m	n.m	Non-MS	–	RIS
37	14	6.0	0.28	0.05	0.07	5.17	0.86	0.13	MS κ	+	RIS
38	6	n.m	8.50	n.m	n.m	4.11	n.m	n.m	Non-MS	–	ON & MOG+
39	9	1.43	5.70	4.00	2.40	3.55	2.50	0.30	Non-MS	–	GBS
40	16.5	1.90	4.54	1.25	0.21	6.40	1.82	0.16	Non-MS		GBS
41	2	0.12	1.82	14.60	8.51	0.57	4.55	0.59	Non-MS	+	RE
42	5.5	4.00	n.m	n.m	n.m	1.67	0.42	0.01	Non-MS	–	RE
43	13	n.m	n.m	n.m	n.m	n.m	n.m	n.m	Non-MS	–	RE
44	6	0.33	1.00	3.00	0.76	0.40	1.19	0.03	MS λ	Inconclusive	Susac
45	5	7.50	n.m	n.m	n.m	8.18	n.m	n.m	Non-MS	Inconclusive	Vasculitis
46	14	n.m	n.m	n.m	n.m	n.m	n.m	n.m	Non-MS	–	Epilepsy
47	3.5	0.1	n.m	n.m	n.m	0.43	3.85	n.d	Non-MS	+	Epilepsy
48	17	0.075	n.m	n.m	n.m	0.3	4.00	n.d	Non-MS	+	ESES
49	17.5	n.m	n.m	n.m	n.m	27.30	n.m	n.m	Non-MS	–	Hashimoto
50	8.5	n.m	n.m	n.m	n.m	15.30	n.m	n.m	Non-MS	–	Sydenham chorea
51	1	n.m	n.m	n.m	n.m	n.m	n.m	n.m	Non-MS	–	Monoplegia left extremity
52	15	2.31	2.35	1.02	1.53	10.82	n.m	0.95	Non-MS	Inconclusive	CADASIL
53	8.9	n.m	n.m	n.m	n.m	n.m	4.76	n.m	Non-MS	–	Migraine
54	17.5	5.00	7.80	1.55	1.14	51.50	n.m	n.m	Non-MS	–	Migraine
55	14.5	n.m	n.m	n.m	n.m	n.m	n.m	n.m	Non-MS	–	Migraine
56		n.m	n.m	n.m	n.m	n.m	n.m	n.m	Non-MS	–	Observation

OCB test positive (+), presence of ≥2 bands in CSF, and their absence in serum; OCB test negative (–), absence of oligoclonal bands in CSF; OCB test is inconclusive, a very weak immunostaining intensity of bands. n.m., not measurable, very low immunoreactivity (none or trace) of FLC bands; n.d., not done; n.c., not calculated, very high immunoreactivity of FLC bands.

Taken together, the FLC patterns in seven out of 35 non-MS cases were close to normal (i.e. similar to the negative control): CIS (n = 2), RE (n = 1), epilepsy (n = 1), monoplegia of left upper extremity (n = 1), migraine (n = 1), non-MS case under observation (n = 1). Mild/moderate abnormalities of FLC patterns were observed in other non-MS patients (n = 16), which included patients with CIS (n = 3), TMY (n = 2), GBS (n = 2), RIS (n = 1), migraine (n = 1), Sydenham's chorea (n = 1), Hashimoto disease (n = 1), ADEM (n = 1), ON & MOG+ (n = 1), RIS (n = 1) and CADASYL (n = 1). Nine out of 35 non-MS cases demonstrated highly abnormal FLC patterns which, however, differed from the FLC patterns in MS: ADEM (n = 2), CIS (1), RE (n = 2), epilepsy (n = 2), NMO (n = 2). In three non-MS cases (Susac syndrome [n = 1], CIS [n = 1] and RIS [n = 1]) the FLC patterns resembled those of MS.

FLC pattern analysis and oligoclonality test versus clinical diagnosis

The FLC diagnostic criteria were applied to support or disfavor the MS diagnosis (Tables 1 and 2). We found that in 19 out of 21 patients with clinically definite MS, the results

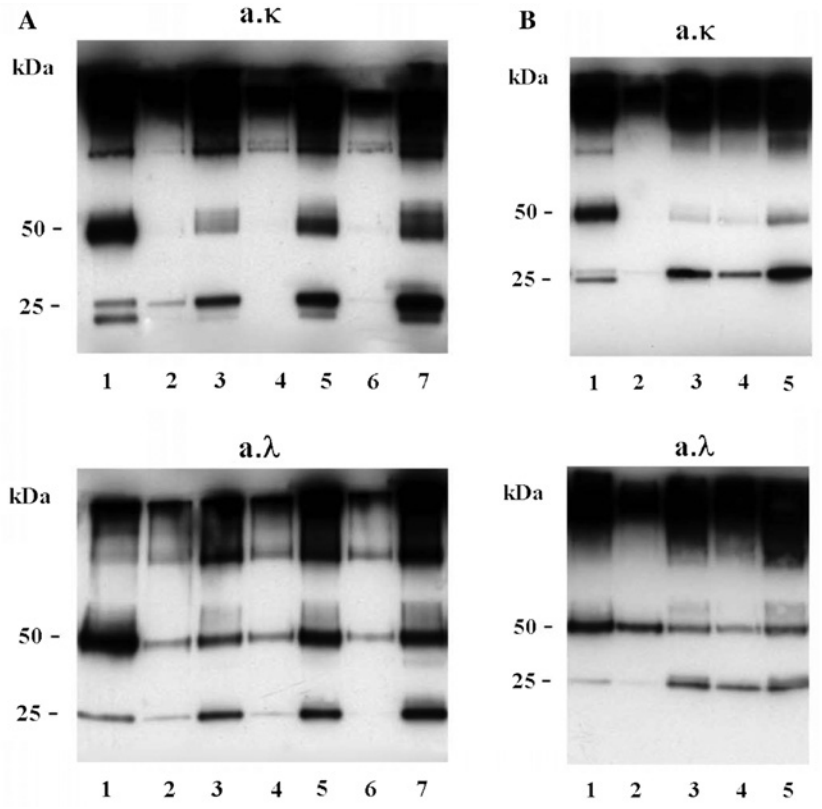

Figure 2: Western blot analysis of the FLC monomer-dimer patterns in the non-MS patients.
FLC bands were detected using anti-κ (a.κ) and anti-λ (a.λ) antibodies. (A) 1 – positive control (MS, CSF); 2 and 3 – TMY (patient #35) CSF and serum, respectively; 4 and 5 – CIS (#23), CSF and serum, respectively; 6 and 7 – Sydenham's chorea (#50) CSF and serum, respectively. (B) 1 – positive control (MS, CSF), 2 and 3 – RE (#42) CSF and serum, respectively, 4 and 5 – NMO (#32) CSF and serum, respectively.

of our FLC analysis supported this diagnosis (sensitivity 90.5%). The results of the oligoclonality test showed that 17 cases were oligopositive, three cases remained inconclusive, and one case was oligonegative (sensitivity 81%).

Our FLC criteria were also applied for the non-MS patient group: 32 out of 35 non-MS cases tested disfavored the MS diagnosis (specificity 91.4%). The oligoclonality test rejected the MS diagnosis in 23 out of 35 cases, in four cases the results were inconclusive, and eight cases were oligopositive (specificity 65.7%).

FLC pattern analysis in MS versus MRI and clinical symptoms

Comparison of the FLC data with those of MRI and clinical assessment at the time of MS diagnosis (Table 3) showed that "mixed type" and "λ type" patients demonstrated a more active onset of the disease as compared to the "κ type" MS patients. Fisher's exact two-tailed test showed significant differences when comparing the MS patient group with "mixed plus λ types" versus that with "κ type" with respect to:

1. amount of brain lesions with a T2 number load >20, $p = 0.015$;
2. amount of active brain lesions with a number of gadolinium enhanced lesions (Gd+) > 3, $p = 0.003$;
3. presence of lesions in spinal cord, $p = 0.009$.

Discussion

The results of our present study have demonstrated the diagnostic importance of FLC monomers and dimers analysis in the diagnosis of pediatric MS. This method showed higher sensitivity (90.5%) and specificity (91.4%) in differentiation of MS from other neurological (non-MS) diseases as compared to the routine oligoclonal bands (OCB) test performed using Sebia agarose gel electrophoresis coupled with immunofixation (81% and 65.7%, respectively). In the future, however, we will check the diagnostic efficiency of our technique against the electrofocusing

Table 3: MS patients: comparison of the FLC patterns with the clinical and MRI data.

Patient #	Sex	Age at onset, years	FLC pattern	Brain MRI at onset		Spine MRI at onset		Presenting symptoms
				T2 load	Number of Gd+ lesions	Presence of lesion	Presence of Gd+ lesions	
1	M	16	κ-type	10	1	No	No	Diplopia, dizziness
2	F	14	κ-type	8	2	No	No	Blurred vision, ptosis, fatigue
3	F	16	κ-type	1	0	C3–C4	No	Optic neuritis
4	F	17	κ-type	>20	0	No	No	Blurred vision
5	F	16	κ-type	10	1[b]	No	No	Optic neuritis
6	M	16	κ-type	1	1	No	No	Diplopia, nystagmus, dizziness
7	M	17	κ-type	15	3	No	No	Diplopia, ptosis, weakness of trochlear nerve
8	F	18	κ-type	14	1	T2–T3	1	Sensory: lt leg. Motor: weakness distal RT leg (4/5). EDSS-2
9	F	10	κ-type	5	0	T10–T11	No	Optic neuritis-Lt eye
10	F	?	κ-type	NA	NA	NA	NA	NA[c]
11	F	11	λ-type	4	3[a]	NA	NA	POLYFOCAL: hemiparesis, central facials, bulbar involvement
12	F	10	λ-type	>20	6	C6	No	Motor/Sensory (leg)
13	F	15	λ-type	>20	5[b]	C1–T2	No	Motor/Sensory (M-distal hand, S-legs)
14	M	15	λ-type	>20	7	C4–C5	Yes	POLYFOCAL: diplopia, blurred vision, weakness of hand (4/5), hypoesthesia
15	M	16.5	λ-type	30	18[b]	C2, C3–C5, T1–T2, T8	No	Diplopia nystagmus
16	F	16	λ-type	3	1	No	No	Dizziness, fatigue
17	F	14	Mixed	>20	10[b]	T11–T12	No	POLYFOCAL: diplopia, blurred vision, nystagmus, dizziness, cerebellar signs
18	F	14	Mixed	>20	5[b]	C2, C4, T3–T4	No	POLYFOCAL: sensory, cerebellar signs, persistent tremor
19	M	7	Mixed	NA	NA	NA	NA	NA

[a]One tumefactive lesion; [b]presence of ring enhancing lesion; [c]NA, no available data.

based OCB analysis which is presently considered as a preferred method to demonstrate oligoclonal Igs in CSF [13].

It is worth mentioning that our Western blot based FLC monomer-dimer pattern analysis is semiquantitative and more laborious compared to the nephelometric FLC assay, which allows quantitative estimation of total FLC levels (monomers plus dimers). On the other hand, our method does not require expensive equipment and can be applied in an ordinary clinical laboratory providing valuable information on FLC pathology which cannot be obtained by nephelometry. We believe that these two techniques of FLC analysis may complement each other, thus further contributing to a precise diagnosis of MS. In addition, by using the nephelometric FLC assay, we can determine the FLC levels at which the electrophoretically resolved FLC bands are weak or unmeasurable and not indicative of MS (see Table 2). This is important from a practical point of view, as in such clear-cut non-MS cases our test may not be necessary.

Our FLC monomer-dimer analysis showed that similarly to our findings in the adult MS population [11], the young MS patients displayed three distinct pathological FLC patterns, characterized by increased levels of κ monomers and dimers and/or λ dimers. The reasons for such differences in the immunological response of MS patients remain unclear. We suppose that these differences may be related to the heterogeneous nature of MS disease, indicating a possible link between FLC parameters and the clinical form of the disease, its severity and progression. Actually, the clinical and MRI data, obtained in this study, indicate the more aggressive onset of the disease in children with "mixed and λ-MS types" than those with "κ-MS type" (Table 3). Longitudinal follow-up of MS patients is needed to understand whether the determination of FLC M-D pattern type could be helpful in predicting the disease course and prognosis and establishing the optimal treatment regiment at an individual patient level.

Comparison of the FLC patterns between CIS and MS patients was of special interest in our study, especially in view of the conflicting data obtained by using the nephelometric FLC assays [14–16]. According to some studies, the FLC levels were similar in these two diseases [14]. Other reports [15, 16], however, showed that the FLC levels in CIS patients were significantly lower than those in MS. Our results were close to those of the latter studies: FLC levels in six out seven of our pediatric CIS patients were low compared to those of MS.

Another important issue is the prognostic value of FLC with respect to conversion of CIS to MS. One of our seven tested pediatric CIS patients showed FLC M-D pattern typical of MS; however, in his case the conversion to MS occurred within a 1-year period. In contrast, CIS patients with the FLC patterns disfavoring MS (six out of seven CIS cases) remained stable over 1–2.5-year period of their follow-up. In this respect it is worth mentioning the results of our 5-year follow-up of eight adult patients, who were diagnosed with CIS at the onset of disease, but showed MS-like FLC patterns. We found that seven of these eight CIS cases converted to MS, and only one of them remained stable (unpublished data). Interestingly, some studies employing nephelometric FLC assay [17, 18] showed that high κ FLC concentration in the CSF might predict the conversion of CIS to MS. In other studies, however, no significant differences in the κ FLC levels were found between the stable CIS patients and those who converted to MS [16]. We suppose that estimation of a single FLC parameter (e.g. κ-FLC only) is insufficient for the evaluation of the prognosis of demyelinating diseases, while the consideration of several FLC parameters (as done in our study) may be more fruitful for this purpose.

Taken together, high CSF levels of FLC per se are indicative of MS, but not sufficient for the precise diagnosis. Our study shows that the determination and combined use of different FLC parameters, including the assessment of FLC dimerization, may be of diagnostic and even of prognostic importance. Our preliminary results showed that the proportion of cases with "λ type" MS was high in our young MS patients (30%) compared to adults (10%) [11]. In fact, the increased λ dimer levels observed in our MS patients with "λ and mixed type" FLC patterns tend to associate with a more aggressive onset of the disease. The pathophysiologic relevance of the increased formation of the covalently-bound FLC dimers is not clear yet, but the structural differences of FLC monomers and dimers imply possible differences in their biological activity [19]. Structurally, FLC dimers resemble Fab region of whole Igs [20], so the binding capabilities of dimers may prevail over that of monomers. The dimeric FLC may interact with B lymphocytes [21] and act as pathogenic miniautoantibodies by binding the factor H [22]. FLC are capable of sensitizing mast cells [23–25], and a possibility was raised that dimerization of FLC is a necessary condition related to cross-linkage of membrane elements [26]. Although precise mechanisms of the enhanced formation of covalent FLC dimers are still not understood in MS, recent findings suggest that both the structural peculiarities of FLC, as well as the disease-related environmental conditions, such as oxidative stress, may play a role [27–29]. Further studies including the structural analysis of FLC and the determination of oxidative stress markers in MS may give insight to these issues and also contribute to the accurate diagnosis of the pediatric MS.

Acknowledgments: We would like to thank Dr. R. Kaplan for performing the statistical analyses and for his invaluable advice and helpful discussion in the preparation of this manuscript.
Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. Boiko A, Vorobeychik G, Paty D, Devonshire V, Sadovnick D, UBC MS Clinic Neurologists. Early onset multiple sclerosis: a longitudinal study. Neurology 2002;59:1006–10.
2. Clerico M, Faggiano F, Palace J, Rice G, Tintorè M, Durelli L. Recombinant interferon beta or glatiramer acetate for delaying conversion of the first demyelinating event to multiple sclerosis. Cochrane Database Syst Rev 2008;CD005278. doi:10.1002/14651858.CD005278.
3. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol 2011;69:292–302.
4. Bastianello S, Pichiecchio A, Spadaro M, Bergamaschi R, Bramanti P, Colonnese C, et al. Atypical multiple sclerosis: MRI findings and differential diagnosis. Neurol Sci 2004;25: S356–60.
5. Rostasy K, Bajer-Kornek B, Venkateswaran S, Hemingway C, Tardieu M. Differential diagnosis and evaluation in pediatric inflammatory demyelinating disorders. Neurology 2016;87 (Suppl 2):S28–37.
6. Chabas D, Ness J, Belman A, Yeh EA, Kuntz N, Gorman MP, et al. Younger children with MS have a distinct CSF inflammatory profile at disease onset. Neurology 2010;74:399–405.
7. Fischer C, Arneth B, Koehler L, Lotz J, Lackner KJ. Kappa free light chains in cerebrospinal fluid as markers of intrathecal immunoglobulin synthesis. Clin Chem 2004;50:1809–13.
8. Desplat-Jego S, Feuillet L, Pelletier J, Bernard D, Cherif A, Boucraut J. Quantification of immunoglobulin free light chains in cerebrospinal fluid by nephelometry. J Clin Immunol 2005;25:338–45.
9. Presslauer S, Milosavljevic D, Brucke T, Bayer P, Hubl W. Elevated levels of kappa free light chains in CSF to support the diagnosis of multiple sclerosis. J Neurol 2008;255:1508–14.
10. Kaplan B, Aizenbud BM, Golderman S, Yaskariev R, Sela BA. Free light chain monomers in the diagnosis of multiple sclerosis. J Neuroimmunol 2010;229:263–73.
11. Kaplan B, Golderman S, Yahalom G, Yeskaraev R, Ziv T, Aizenbud BM, et al. Free light chain monomer-dimer patterns in the diagnosis of multiple sclerosis. J Immunol Methods 2013;390:74–80.
12. Kaplan B, Golderman S, Sela B. Immunoglobulin free light chains in the diagnosis of multiple sclerosis. In: Scholz E, Muller C, editors. Multiple sclerosis: causes, diagnosis and management. Hauppauge, New York: Nova Science Publishers, 2012:131–42.
13. Gastaldi M, Zardini E, Franciotta D. An update on the use of cerebrospinal fluid analysis as a diagnostic tool in multiple sclerosis. Expert Rev Mol Diagn 2017;17:31–46.
14. Passerini G, Dalla Costa G, Sangalli F, Moiola L, Colombo B, Locatelli M, et al. Free light chains and intrathecal B cells activity in multiple sclerosis: a prospective study and meta-analysis. Mult Scler Int 2016;2016:2303857.
15. Makshakov G, Nazarov V, Kochetova O, Surkova E, Lapin S, Evdosheko E. Diagnostic and prognostic value of the cerebrospinal fluid concentration of immunoglobulin free light chains in clinically isolated syndrome with conversion to multiple sclerosis. PloS One 2015;10:e0143375.
16. Presslauer S, Milosavljevic D, Huebl W, Parigger S, Schneider-Koch G, Bruecke T. Kappa free light chains: diagnostic and prognostic relevance in MS and CIS. PloS One 2014;9:e89945.
17. Villar LM, Espiño M, Costa-Frossard L, Muiel A, Jiménez J, Alvarez-Cermeño JC. High levels of cerebrospinal fluid free kappa chains predict conversion to multiple sclerosis. Clin Chim Acta 2012;413:1813–6.
18. Menéndez-Valladeres P, Garcia-Sánchez M, Cuadri Benítez P, Lucas M, Adorna Martínez M, Carranco Gallán V, et al. Free kappa chains in cerebrospinal fluid as a biomarker to assess risk conversion to multiple sclerosis. Mult Scler J Exp Transl Clin 2015:1–9; doi: 10.1177/2055217315620935.
19. Kaplan B, Livneh A, Sela BA. Immunoglobulin free light chain dimers in human diseases. Scientific World J 2011;11:726–35.
20. Schiffer M, Girling RL, Ely KR, Edmundson AB. Structure of lambda-type Bence-Jones protein at 3.5 a resolution. Biochemistry 1973;12:4620–31.
21. Wall JS, Ayoub FM, O'Shea PS. A study of the interactions of an immunoglobulin light chain with artificial and B-lymphocyte membranes. Front Biosci 1996;1:a46–58.
22. Jokiranta TS, Solomon A, Pangburn MK, Zipfel PF, Meri S. Nephritogenic lambda light chain dimer: a unique human miniautoantibody against complement factor H. J Immunol 1999;163:4590–6.
23. Rocken M, Hultner L. Heavy functions for light chains. Nat Med 2002;8:668–70.
24. Redegeld FA, Nijkamp FP. Immunoglobulin free light chains and mast cells: pivotal role in T-cell mediated reactions? Trends Immunol 2003;24:181–5.
25. Thio M, Blokhuis BR, Nijkamp FP, Redegeld FA. Free immunoglobulin light chains: a novel target in the therapy of inflammatory diseases. Trends Pharmacol Sci 2008;29: 170–4.
26. Kaplan AP. A new mechanism for immunologic initiation of asthma. Proc Natl Acad Sci USA 2005;102:1267–8.
27. Wilkinson B, Gilbert H. Protein disulfide isomerase. Biochim Biophys Acta 2004;1699:35–44.
28. Xiao R, Lundstrom-Ljung J, Holmgren A, Gilbert HF. Catalysis of thio/disulfide exchange. J Biol Chem 2005;280:21099–106.
29. Ferretti G, Bacchetti T, DiLudovico F, Viti B, Angeleri VA, Danni M, et al. Intracellular oxidative activity and respiratory burst of leucocytes isolated from multiple sclerosis patients. Neurochem Int 2006;48:87–92.