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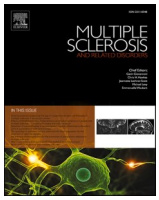

Chloride intracellular channel protein-1 (CLIC1) antibody in multiple sclerosis patients with predominant optic nerve and spinal cord involvement

Zerrin Karaaslan [a,b], Büşra Şengül-Yediel [d], Hande Yüceer-Korkmaz [a,b], Elif Şanlı [a,b], Duygu Gezen-Ak [d], Erdinç Dursun [d], Özlem Timirci-Kahraman [e], Ahmet Tarık Baykal [f], Vuslat Yılmaz [b], Recai Türkoğlu [c], Murat Kürtüncü [g], Tuncay Gündüz [g], Yasemin Gürsoy-Özdemir [h], Erdem Tüzün [b], Cem İsmail Küçükali [b,*]

[a] Institute of Graduate Studies in HealthySciences, Istanbul University, Istanbul, Turkey
[b] Department of Neuroscience, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey
[c] Department of Neurology, Istanbul Haydarpasa Numune Training and Research Hospital, Istanbul, Turkey
[d] Department of Neuroscience, Institute of Neurological Sciences, Istanbul University-Cerrahpasa, Istanbul, Turkey
[e] Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey
[g] Department of Medical Biochemistry, Faculty of Medicine, Acibadem University, Istanbul, Turkey
[f] Department of Neurology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey
[h] Department of Neurology, School of Medicine, Koç University, Istanbul, Turkey

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ABSTRACT

Introduction: Antibodies to cell surface proteins of astrocytes have been described in chronic inflammatory demyelinating disorders (CIDD) of the central nervous system including multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD). Our aim was to identify novel anti-astrocyte autoantibodies in relapsing remitting MS (RRMS) patients presenting predominantly with spinal cord and optic nerve attacks (MS-SCON).

Methods: Sera of 29 MS-SCON patients and 36 healthy controls were screened with indirect immunofluorescence to identify IgG reacting with human astrocyte cultures. Putative target autoantigens were investigated with immunoprecipitation (IP) and liquid chromatography-mass/mass spectrometry (LC-MS/MS) studies using cultured human astrocytes. Validation of LC-MS/MS results was carried out by IP and ELISA.

Results: Antibodies to astrocytic cell surface antigens were detected in 5 MS-SCON patients by immunocytochemistry. LC-MS/MS analysis identified chloride intracellular channel protein-1 (CLIC1) as the single common membrane antigen in 2 patients with MS-SCON. IP experiments performed with the commercial CLIC1 antibody confirmed CLIC1-antibody. Home made ELISA using recombinant CLIC1 protein as the target antigen identified CLIC1 antibodies in 9/29 MS-SCON and 3/15 relapsing inflammatory optic neuritis (RION) patients but in none of the 30 NMOSD patients, 36 RRMS patients with only one or no myelitis/optic neuritis attacks and 36 healthy controls. Patients with CLIC1-antibodies showed trends towards exhibiting reduced disability scores.

Conclusion: CLIC1-antibody was identified for the first time in MS and RION patients, confirming once again anti-astrocytic autoimmunity in CIDD. CLIC1-antibody may potentially be utilized as a diagnostic biomarker for differentiation of MS from NMOSD.

1. Introduction

Multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD) are chronic demyelinating inflammatory disorders of the central nervous system and potential causes of permanent disability (Kuchling & Paul, 2020); (Akaishi et al., 2020). Once considered a variant of MS, NMOSD has later been recognized as a distinct entity, characterized with astrocyte loss and subsequent demyelination caused

* Corresponding author.
E-mail address: cemsmile@istanbul.edu.tr (C. İsmail Küçükali).

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by anti-aquaporin-4 (Aqp-4) antibodies in the majority of the patients (de Seze et al., 2016). Astrocyte antibodies are not only found in NMOSD patients but also in other inflammatory demyelinating diseases such as MS and relapsing inflammatory optic neuritis (RION). As an example, antibodies directed against other astrocytic cell surface antigens such as Kir4.1 and Aqp-1 have been identified in NMOSD and MS patients (Berger & Reindl, 2015); (Tuzun et al., 2014). This is not unexpected, given the significance of glial cells in the maintenance of neurons and oligodendrocytes. Notably, Aqp-1 antibodies recognizing the extracellular part of the protein are found predominantly in MS patients with myelitis (Tzartos et al., 2013). Moreover, we have recently identified antibodies to Sox2 expressing glial cells of optic nerves in patients with RION (Erdag et al., 2023). Thus, intriguingly, well-characterized astrocytic antibodies are often found in demyelinating inflammatory disorders presenting with optic nerve and/or spinal cord involvement.

While most MS patients present with attacks afflicting supratentorial cerebellar hemispheres, cerebellum, brainstem, as well as the spinal cord and optic nerves, a fraction of MS patients may present only or predominantly with optic neuritis and myelitis attacks. Although MS patients with or without spinal cord and optic nerve involvement appear to present with similar clinical, neuropsychological, and immunological features (Sen et al., 2021), an in-depth anti-neural antibody analysis of this MS subgroup has never been performed. To investigate the presence of novel astrocytic cell surface autoantibodies in this subgroup of MS, we first screened sera of patients by immunocytochemistry and then subjected the serum IgG-human astrocyte immunoprecipitates of these patients to mass spectrometry analysis. Our findings provide preliminary evidence regarding the presence of astrocytic cell membrane antibodies in MS patients with myelitis and optic neuritis attacks.

2. Methods

2.1. Participants

This study includes 29 consecutively enrolled relapsing remitting MS (RRMS) patients presenting with predominant optic nerve and spinal cord involvement (MS-SCON). All MS-SCON patients satisfied the revised McDonald's 2017 criteria for clinically definite MS (Thompson et al., 2018), presented with at least one optic neuritis and one myelitis attack, displayed at least four attacks during the disease course, had not experienced any long extensive transverse myelitis attacks, were negative for Aqp-4 and MOG antibodies and did not satisfy the NMOSD criteria (Wingerchuk et al., 2015). In MS patients, optic neuritis attack was defined as acute onset visual loss in one or both eyes and myelitis attack was defined as acute onset loss of motor and/or sensory functions in both lower or all four limbs (with or without bladder, bowel or sexual dysfunction) in parallel with hyperintense lesions congruent with clinical findings on T2-weighted spinal cord images. Although 20 MS-SCON patients had additional clinical features indicating brainstem, cerebellum, or supratentorial hemisphere involvement (MS-SCON+), number of such attacks was less than half of the total number of MS attacks (1 or 2 attacks/patient). Remaining 9 MS-SCON patients had only experienced spinal cord and optic nerve attacks (MS-SCON). As control groups, 36 conventional RRMS (CMS) patients fulfilling the McDonald's 2017 criteria for clinically definite MS, 30 NMOSD patients (19 with Aqp-4 antibodies) fulfilling the relevant criteria, 15 RION patients and 36 healthy individuals were included. CMS patients were RRMS patients with a medical history of at least 4 attacks. Fifteen patients had experienced neither optic neuritis nor myelitis attacks (CMS), 10 patients had experienced only one optic nerve (CMS-ON) and 11 patients had experienced only one spinal cord attack (CMS-S). None of the 36 CMS patients had both optic nerve and spinal cord attacks in their medical history. The patients with RION had normal cranial and spinal magnetic resonance imaging, chest X-ray, blood biochemistry, total blood count, vitamin B12 and folate levels and were negative for thyroid antibodies, anti-nuclear antibodies, Aqp-4 and MOG antibodies. Only RION patients with three or more isolated optic neuritis episodes were included. All included patients were in remission and none of the patients were under treatment with immunosuppressive or immunomodulation medications. Demographic features, age of disease onset, duration of disease, EDSS scores, progression index (EDSS divided by disease duration) values and oligoclonal band (OCB) types of all patients were recorded (Table 1). The study was approved by the local ethics committee and written informed consent was obtained from all participants.

2.2. Human astrocyte cultures and immunocytochemistry

Primary fetal human astrocytes were obtained from ScienCell Research Laboratories (Lonza, Walkersville, MD, USA). Astrocytes were cultured in Clonetics AGM BulletKit (Lonza), containing astrocyte basal medium, rhEGF, insulin, ascorbic acid, L-glutamine and FBS. Cells were subcultured when they reached 70–80% confluency and were seeded to six-well plates and incubated at 37 °C and 5% CO_2 in a humidified chamber. All experiments were carried out with a passage number of less than four (Sengul et al., 2021).

Cultured astrocytes were incubated with serum samples at 1:100 dilution in DMEM containing 5% BSA for 1 h at room temperature. After washing, cells were fixed with 4% PFA in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min and incubated with rabbit anti-human glial fibrillary acidic protein (GFAP, 1:500, Abcam, Cambridge, UK) for 1 h at room temperature. Immunoreactivities were visualized following 45 min incubation with DyLight 488-conjugated goat anti-human IgG (Abcam) and Alexa Fluor 548-conjugated goat anti-rabbit IgG (Abcam) for human IgG and GFAP, respectively. Immunostaining was evaluated by Leica DMIL inverted fluorescence microscope and the Leica Application Suite Image Overlay Software (Leica Microsystems Ltd., Heerbrugg, Switzerland). Moderate to strong DyLight 488-conjugated anti-human IgG-induced green color that co-localized with Alexa Fluor 548-conjugated anti-GFAP-induced red color was considered as positive. IgG immunoreactivity of sera was also investigated using cultured rat cortical neurons, as described previously (Kucukali et al., 2022).

2.3. Immunoprecipitation

Cultured human astrocytes were used for immunoprecipitation with participants' sera. The cells were plated in DMEM media containing 2 mM L-glutamine, 10% FBS and were incubated at 37 °C and 5% CO_2 in a

Table 1
Clinical and demographic features of patient subgroups.

	MS-SCON (n=29)	CMS (n=36)	RION (n=15)	NMOSD (n=30)	HC (n=36)
Age	36.7±9.3	29.6 ±8.8	42.8 ±11.8	45.7±10.4	32.8 ±8.3
Gender (women/ men)	14/15	24/12	9/6	24/6	26/10
Age of disease onset	27.1±9.2	26.4 ±7.8	34.5 ±10.5	34.2±13.3	NA
Disease duration (years)	10.0±7.8	8.0±6.5	8.5±6.3	12.3±9.0	NA
EDSS	2.5±1.6	1.7±1.0	2.1±1.1	3.5±2.0	NA
Progression index	0.5±0.7	0.3±0.3	0.4±0.4	1.0±0.6	NA
CSF OCB+ (Type 2 or 3)	21/29	27/36	0/15	5/30	NA

Numeric data are given as mean ± standard deviation.
HC, healthy controls; MS-SCON, multiple sclerosis presenting predominantly with spinal cord and optic nerve attacks; CMS, conventional multiple sclerosis; RION, relapsing inflammatory optic neuritis; NMOSD, neuromyelitis optica spectrum disorder; NA, not applicable; EDSS, expanded disability status scale; CSF, cerebrospinal fluid; OCB, oligoclonal bands.

humidified atmosphere. Immunoprecipitation was performed on astrocytes (2×10^7 cells per sample) that were grown in 100 mm petri dishes. After the "pre-clear" procedure, the sera were added to the cell lysates and incubated overnight at +4 °C. Following centrifugation and washing, the supernatant was removed and the pellet was stored. The pellet was washed, treated with lysis buffer for 45 min at 4 °C, centrifuged for 15 min at 8000 g and the supernatants were incubated with protein A/G agarose beads (Pierce, Rockford, IL, USA) overnight at 4 °C. After centrifugation, the beads were washed, re-suspended in Laemmli buffer, boiled for 10 min and analyzed by 4–15% SDS-PAGE. Bands that were only obtained with sera of MS-SCON patients but not healthy controls were extracted. Only bands that included astrocytic cell surface antigens and were present in more than 1 patient were subjected to further investigation (Kucukali et al., 2022).

2.4. LC-MSMS analysis

To the protein mixture DTT was added to 10 mM final concentration and incubated at 60℃ for 10 min. The mixture was alkylated in dark for 30 min with 20 mM IAA. To the resulting mixture 1 ug of sequencing grade trypsin (Promega Gold) was added and incubated at 55℃ for 1.5 h. The digest was acidified with 1 ul formic acid and transferred to an LC vial for injection. The samples were analyzed by the protocols in our previous studies (Gurel et al., 2019). Briefly, tryptic peptides were trapped on a Symmetry C18 (5 μm,180 μm i.d. × 20 mm) column and eluted with ACN gradient (4% to 40% ACN, 0.3 ul/min flow rate) with a total run time of 60 min on a CSH C18 (1.7 μm, 75 μm i.d. × 250 mm) analytical nano column. Data were collected in positive ion sensitivity mode using a novel data-independent acquisition mode (DIA) coined as SONAR (Moseley et al., 2018) with a quadrupole transmission width of 24 Da. Progenesis-QIP (V.2.4 Waters) was used for data processing.

2.5. Validation assays

Since CLIC1 is ubiquitously expressed by a wide range of non-neuronal tissue including human embryonic kidney 293 (HEK293) and Chinese hamster ovary (CHO) cells (Dai et al., 2022), (Alanazi et al., 2020) widely used in molecular assays, seropositive MS-SCON sera showed reactivity with both transfected and non-transfected HEK293 and CHO cells (not shown). Thus, cell-based assay could not be used for validation. In immunoprecipitation experiments performed for validation of chloride intracellular channel protein-1 (CLIC1) antibody, human astrocyte cell extracts were prepared using 1 mL of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, %1 Triton X-100, 1:100 protease inhibitors). Twenty microliters of patients' sera were added to the soluble fraction, followed by incubation for overnight at 4 °C. The samples were added to 50 μl of protein G-Sepharose (Sigma-Aldrich) and incubated for 4 h at 4 °C. The immunoprecipitate was washed three times with PBS, electrophoresed on SDS-PAGE and transferred to nitrocellulose membrane, which was incubated with the commercial antibody to CLIC1 (PA5–109,552, Thermo Fisher, Waltham, MA, USA) at 1:500 dilution and the appropriate secondary antibody (Abcam). The membranes were visualized by MicroChemi 4.2 MP (DNR Bio-Imaging Systems, Neve Yamin, Israel). Immunoprecipitates incubated with a commercial antibody to an irrelevant protein actin (Abcam) or with only the seconday anti-IgG antibody served as negative controls.

A home made ELISA was designed for further verification of the CLIC1 antibody in a larger patient cohort. The purified recombinant human CLIC1 protein (1 mg/ml, Abcam) was coated onto 96-well microtiter plates in 0.1 M carbonate/bicarbonate buffer overnight at 4 °C. Sera (1:100) were added and plates were incubated overnight at 4 °C. After washing, horseradish peroxidase (HRP)-conjugated anti-human IgG (Abcam) (1:1000) was added and plates were then incubated at room temperature for 1 h, followed by washing and incubation with a chromogen solution in the presence of H_2O_2. Plates were read at a wavelength of 450 nm and results were expressed as optical densities (OD). A commercial anti-CLIC1 antibody (PA5–109,552, Thermo Fisher) was used as a positive control. OD values were converted to pg/μl by comparison against a standard curve of serial dilutions of the commercial anti-CLIC1 antibody (Thermo Fisher) incubated in ELISA wells. The cut-off level for CLIC1 antibody positivity (48.3 pg/μl) was defined as two standard deviations above the mean of healthy controls.

2.6. Statistical Analysis

Due to abnormal distribution of data (as assessed by Kolmogorov-Smirnov test), non-parametric tests were used for comparison of groups, which was conducted by Mann-Whitney U or Kruskal-Wallis (and post-hoc Dunn) tests, as required. Categorical parameters were compared with chi-square test. p value below 0.05 was defined as statistical significance.

3. Results

3.1. Identification of MS-SCON patients with astrocyte surface antibodies

To characterize serum antibodies directed against surface antigens of astrocytes, we utilized a specific immunocytochemistry method. Serum incubations were done before fixation and permeabilization steps to eliminate detection of antibodies reactive with intracellular antigens. This method identified serum IgG immunoreacting with cell surface antigens of astrocytes in 5 of 29 MS-SCON patients and none of the 36 healthy controls. Immunoreactivities of serum IgG and GFAP antibodies significantly co-localized, confirming the astrocytic specificity of detected antibodies. Serum IgG of 5 seropositive MS-SCON patients showed a reactivity pattern and intensity that were highly reminiscent of serum IgG of Aqp-4 antibody positive NMOSD patients, used as positive control (Fig. 1). Immunocytochemistry assays performed with primary rat cortical neurons did not yield positive results in any of the MS-SCON patients and controls (not shown).

3.2. Identification of CLIC1 antibodies

To further characterize the target antigens of astrocyte-reactive IgG, immunoprecipitation studies were conducted with cultured human astrocytes using sera of 5 astrocyte-reactive MS-SCON patients, 5 non-astrocyte reactive MS-SCON patients and 5 healthy controls. SDS-PAGE analysis of immunoprecipitation pellets revealed a common band in 2 MS-SCON patients but not in any of the healthy controls or remaining 8 MS-SCON patients. The LC-MS/MS analysis of these bands yielded a total of 94 proteins discriminating between MS-SCON patients and healthy controls. There was only one sequence (Uniprot Protein Accession Number, O00299) that was commonly found in samples of 2 astrocyte-reactive MS-SCON patients and also was an astrocytic membrane protein, whereas all other proteins were intracellular antigens. Comparison of this sequence with protein databases yielded CLIC1 as the putative target antigen.

3.3. Validation of CLIC1 antibody positivity and association with clinical features

Serum CLIC1 antibodies were validated by an immunoprecipitation assay and a home made ELISA method. After demonstrating the presence of CLIC1 expression by cultured human astrocytes with preliminary immunoblotting experiments (not shown), sera of 5 astrocyte-reactive MS-SCON patients (including 2 putative CLIC1-antibody positive MS-SCON patients determined by LC-MS/MS), 5 non-astrocyte reactive MS-SCON patients and 5 healthy controls were incubated with human astrocyte lysates. SDS-PAGE analysis of immunoprecipitation pellets with a commercially available CLIC1 antibody revealed a single common band at the same predicted molecular weight (~27 kDa) in 2 MS-SCON patients, but in none of the remaining MS-SCON samples with

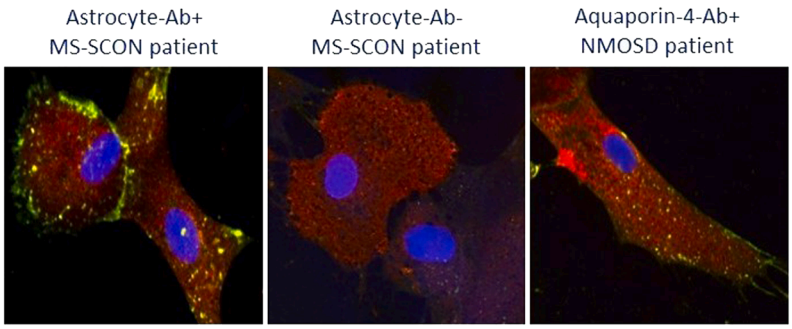

Fig. 1. Immunolabeling of cultured live human astrocytes with serum IgG of MS-SCON (multiple sclerosis presenting predominantly with spinal cord and optic nerve attacks) and aquaporin-4 Ab (antibody) positive NMOSD (neuromyelitis optica spectrum disorder) patients (positive control) using indirect immunofluorescence. The serum IgG of an MS-SCON patient shows reactivity with the membrane of astrocyte cell body (left panel, green) in a pattern similar to serum IgG of seropositive NMOSD patient (right panel). By contrast, serum IgG of another MS-SCON patient does not immunolabel astrocytes (middle panel). Astrocytes were identified by characteristic morphological features and presence of intracellular glial fibrillary acidic protein (GFAP, red). Original magnification (40x), counterstained with DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or without astrocyte-reactivity and healthy control samples (Fig. 2). No bands were observed with the immunoprecipitates of astrocyte-reactive MS-SCON patients, when irrelevant anti-actin antibody was used as the primary antibody or when only secondary anti-IgG antibody was used during immunoblotting.

The home made ELISA performed with samples of all patient and healthy control participants showed CLIC1 antibodies in sera of 9 MS-SCON and 3 RION patients. None of the CMS, NMOSD and healthy controls were found positive (Fig. 3A). Notably, 3 MS-SCON patients showing astrocyte-reactive serum IgG in immunocytochemistry studies but no common immunoprecipitation bands with other MS-SCON patients were negative for CLIC1 antibody. There were no significant differences among patient subgroups and healthy controls by Kruskal-Wallis (p=0.102) and post-hoc Dunn tests. Likewise, comparison of MS-SCON vs MS-SCON+ patients, as well as, CMS vs CMS-S vs CMS-ON patients yielded comparable serum CLIC1 antibody levels (p=0.843). Three of 9 MS-SCON and 6 of 20 MS-SCON+ patients were found to be

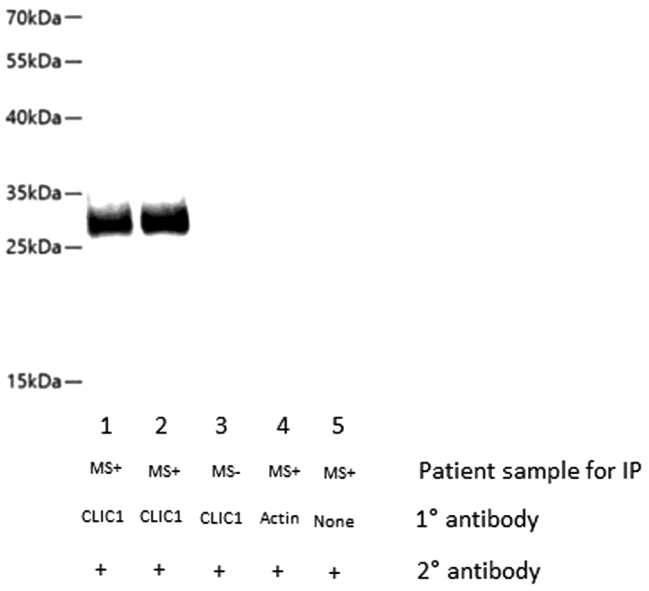

Fig. 2. SDS-PAGE analysis of immunoprecipitates obtained by incubation of astrocyte-reactive (MS+) sera of two separate MS patients with cultured human astrocytes showing a single band at around 27 kDa, as predicted (lanes 1–2). While the commercially available CLIC1 antibody yielded 27 kDa bands with immunoprecipitates of astrocyte-reactive MS-SCON patients (lanes 1–2), no reactivity was observed with the immunoprecipitate of a non-astrocyte-reactive MS-SCON patient (lane 3). An irrelevant commercial antibody to actin did not yield bands with the immunoprecipitates of the astrocyte-reactive MS-SCON patients (MS+) (lane 4). Likewise, incubation of serum-astrocyte immunoprecipitates with secondary antibody only in the absence of a primary antibody did not yield any bands (lane 5).

CLIC1 antibody positive by indirect ELISA (Fig. 3B).

CLIC1 antibody positive MS-SCON and RION patients showed comparable age, gender, age of disease onset, disease duration, progression index and OCB positivity rates than CLIC1 antibody negative patients. However, both seropositive MS-SCON and RION patients showed trends toward displaying reduced EDSS scores (Tables 2 and 3).

4. Discussion

In this study, we have identified serum antibodies to CLIC1, expressed by both nucleus and cell membrane (Valenzuela et al., 1997, Tulk et al., 2002, Littler et al., 2004), for the first time in MS-SCON and RION patients. Similar to previously described anti-astrocytic antibodies Aqp-4, Aqp-1 and Kir4.1, target antigen of CLIC1-antibodies is located on the cell membrane suggesting that these antibodies may have pathogenic action. As a matter of fact, Aqp-4 and Kir4.1 antibodies have been shown to reduce the expression levels of their target antigens in rodent models putatively through enhanced endocytosis and complement-mediated membrane destruction (Srivastava et al., 2012); (Saadoun et al., 2010). Similarly, reduced cerebral Aqp-1 expression has been reported in areas of demyelination in an Aqp-1 antibody positive NMOSD patient (Turkoglu et al., 2017). Whether CLIC1-antibodies employ a similar action on their target antigen needs to be further studied.

The initial immunocytochemistry studies confirmed the presence of serum IgG interacting with the cell membrane astrocytes in 5 MS-SCON sera negative for Aqp-4 antibodies. Although CLIC1-antibodies were identified in two of these patients by mass spectrometry analysis and confirmatory assays, these antibodies could not be found in the remaining 3 MS-SCON patients' sera harboring astrocyte-reactive IgG. Thus, the immunoreactivity observed by immunocytochemistry studies might not necessarily be an indicator of anti-CLIC1 IgG but it might rather be pointing serum IgG directed against putative additional astrocytic membrane antigens, which are required to be explored in future studies.

CLIC1 is expressed by astrocytes and astrocytic tumors (Wang et al., 2021); (Skaper, 2011). Its translocation to the plasma membrane is indicative of conversion of astrocytes into the pro-inflammatory A1 form and induction of neurotoxicity through the generation of reactive oxygen species (Skaper, 2011). Cerebrospinal fluid levels of CLIC1 are elevated in MS and correlate with disease severity (Masvekar et al., 2019). Given the lower EDSS scores of MS patients with CLIC1-antibody, it is tempting to speculate that CLIC1-antibody is produced as a compensatory measure to counterbalance the pro-inflammatory action of A1 astrocytes. Presence of CLIC1 antibodies in MS-SCON but not MS patients with restricted involvement of optic nerve and spinal cord implies distinct pathophysiological mechanisms in these two clinically-defined MS subgroups. In the earlier stage, differential diagnosis of MS with recurrent myelitis and optic neuritis attacks from NMOSD may prove difficult, especially because Aqp-4 antibody positive NMOSD may occasionally manifest with short myelitis attacks

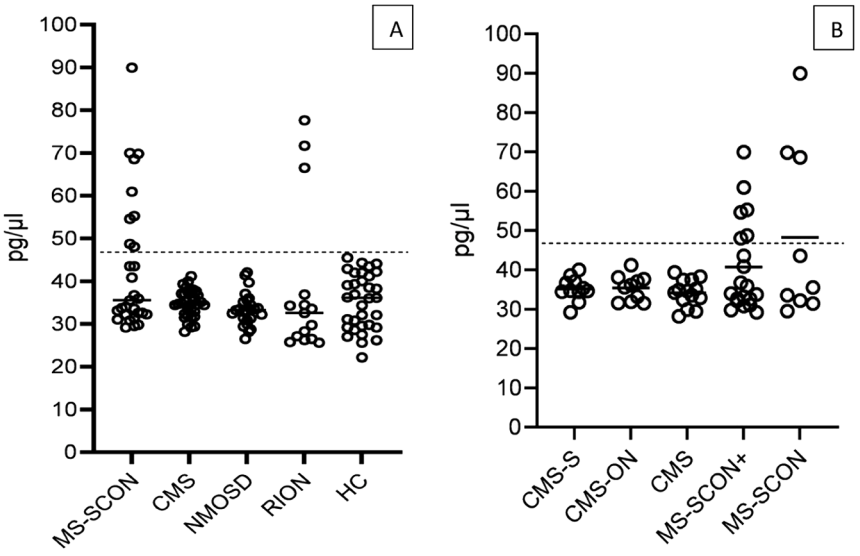

Fig. 3. Horizontal lines indicate the mean value of each group. (A) ELISA detection of IgG antibodies directed against chloride intracellular channel protein 1 (CLIC1) in sera of patients with multiple sclerosis presenting predominantly with spinal cord and optic nerve attacks (MS-SCON), conventional multiple sclerosis (CMS), relapsing inflammatory optic neuritis (RION), neuromyelitis optica spectrum disorder (NMOSD) and healthy controls (HC). (B) Serum levels of CLIC1 antibodies were comparable among CMS patients with at least one spinal cord (CMS-S) or optic nerve (CMS-ON) attack in medical history versus CMS patients with no spinal cord or optic nerve attacks (CMS). Likewise, MS-SCON patients with (MS-SCON+) versus without (MS-SCON) additional non-spinal cord or non-optic nerve attacks had comparable levels of CLIC1 antibodies. The dashed lines represent 2 standard deviations (2SD) above the mean of the healthy control samples (cut-off values for positivity).

Table 2
Comparison of MS-SCON patients with and without CLIC1-Ab.

	CLIC1-Ab+ MS-SCON (n=9)	CLIC1-Ab- MS-SCON (n=20)	p value
Age	36.0±8.9	37.0±9.7	0.389
Gender (women/men)	4/5	10/10	0.782
Age of disease onset	28.6±8.0	26.5±9.8	0.279
Disease duration (years)	8.3±7.5	10.7±8.1	0.249
EDSS	1.8±0.7	2.9±1.8	0.023
Progression index	0.5±0.5	0.6±0.7	0.363
CSF OCB+ (Type 2 or 3)	7/9	14/20	0.665

Numeric data are given as mean ± standard deviation.
MS-SCON, multiple sclerosis presenting predominantly with spinal cord and optic nerve attacks; Ab, antibody; CLIC1, chloride intracellular channel protein 1; EDSS, expanded disability status scale; CSF, cerebrospinal fluid; OCB, oligoclonal bands.

Table 3
Comparison of relapsing inflammatory optic neuritis (RION) patients with and without CLIC1-Ab.

	CLIC1-Ab+ RION (n=3)	CLIC1-Ab- RION (n=12)	p value
Age	42.6±15.9	42.9±11.4	0.490
Gender (women/men)	2/1	7/5	>0.999
Age of disease onset	39.0±14.7	33.4±9.7	0.293
Disease duration (years)	4.0±2.6	9.6±6.5	0.125
EDSS	1.9±1.2	2.7±0.6	0.082
Progression index	0.3±0.3	0.9±0.6	0.105

Numeric data are given as mean ± standard deviation.
Ab, antibody; CLIC1, chloride intracellular channel protein 1; EDSS, expanded disability status scale.

(Flanagan et al., 2015); (Zhang et al., 2017). Therefore, a biomarker that pinpoints the diagnosis of MS is highly required. Whether CLIC1-antibody may serve this purpose will be determined by future studies conducted with larger patient numbers.

Since CLIC1 is abundantly expressed by cell lines commonly used for cell-based assays (Dai et al., 2022); (Alanazi et al., 2020), validation assays were done with IP and ELISA methods. As a limitation of these assays, antibodies directed against intracellular epitopes, which are not expected to possess pathogenic action, were also determined. This might be one of the reasons underlying increased prevalence of seropositivity by ELISA as compared to immunocytochemistry in the MS-SCON cohort. Therefore, IP and ELISA methods utilizing the extracellular region of CLIC1 may provide a more functionally relevant account of CLIC1-antibodies.

Overall, our results lend further support for the presence of anti-astrocytic autoimmunity in chronic inflammatory demyelinating disorders of the brain. Future research focused on the functional features and diagnostic value of CLIC1-antibody and investigation of additional novel astrocytic antibodies is expected to shed further light and provide a more accurate account on the role of astrocytes in these disorders.

Ethics approval and consent to participate

Written informed consent was obtained from all participants and study was approved by Clinical Research Ethics Committee of the Koç University, Faculty of Medicine (30.05.2016–2016.123.IRB2.077).

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CRediT authorship contribution statement

Zerrin Karaaslan: Conceptualization, Investigation, Formal analysis, Methodology, Validation, Visualization. **Büşra Şengül-Yediel:** Investigation, Formal analysis, Methodology, Validation, Visualization. **Hande Yüceer-Korkmaz:** Investigation, Methodology. **Elif Şanlı:** Investigation, Methodology, Formal analysis, Validation. **Duygu Gezen-Ak:** Conceptualization, Investigation, Formal analysis, Methodology, Visualization. **Erdinç Dursun:** Conceptualization, Investigation, Formal analysis, Methodology, Visualization. **Özlem Timirci-Kahraman:** Data curation, Formal analysis, Methodology. **Ahmet Tarık Baykal:** Data curation, Formal analysis, Methodology. **Vuslat Yılmaz:** Conceptualization, Formal analysis, Methodology, Validation, Visualization, Writing – original draft. **Recai Türkoğlu:** Investigation, Methodology. **Murat Kürtüncü:** Conceptualization, Investigation, Methodology. **Tuncay Gündüz:** Conceptualization, Investigation, Methodology. **Yasemin Gürsoy-Özdemir:** Conceptualization, Investigation, Methodology. **Erdem Tüzün:** Conceptualization, Data curation, Formal

analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft. **Cem İsmail Küçükali:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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