Cerebrospinal fluid antibodies to aquaporin-4 in neuromyelitis optica and related disorders: frequency, origin, and diagnostic relevance

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of AIs allows quantification of antigen-specific AQP4-specific antibody index (AI), AIAQP4. Calculation of the AI, a cut-off of 4 has been recommended [15,16]. Briefly, AIAQP4 values were calculated as the ratio between the CSF/serum quotient for AQP4-IgG, Q_AQP4, and the CSF/serum quotient for total IgG, Q_IgG, i.e., AIAQP4 = Q_AQP4/Q_IgG. If AQP4-IgG are produced intrathecally, Q_AQP4 would exceed Q_IgG, resulting in AI values >1. Usually, values >1.5 are considered as evidence of intrathecal specific antibody synthesis [15,16]. However, if titres instead of concentrations are used to calculate the AI, a cut-off of 4 has been recommended [17]. Reiber’s empiric hyperbolic function Q_{lim} was applied to control for possible underestimation of intrathecal specific synthesis due to disturbances of the blood-CSF barrier function:[18]

\[
Q_{\text{lim(IgG)}} = 0.93 \sqrt{(Q_{\text{Al}})^2 + 6 \times 10^{-6} - 1.7 \times 10^{-3}}
\]

In case of Q_{IgG} > Q_{\text{lim(IgG)}}, AIAQP4 was calculated as the ratio between Q_AQP4 and Q_{lim(IgG)}, i.e., AIAQP4 = Q_AQP4/Q_{lim(IgG)}. For assessment of the AI, serum samples were tested at 1:10, 1:100, 1:500, 1:1,000, and 1:5,000 dilutions, and at 1:25, 1:50, 1:62.5, 1:75, 1:125, 1:250, 1:750, 1:1250, 1:1750, 1:2000, 1:2500, 1:3000, 1:4000, 1:6000, 1:7000, 1:8000, 1:9000, and 1:10,000 dilutions, where applicable. CSF samples were tested undiluted, at 1:10 dilution, and, in addition, at dilutions that would indicate intrathecal production as defined by an elevated AQP4-AI of >4.

Values for Q_{IgG} exceeding the hyperbolic discrimination line, Q_{lim}, or detection of CSF-restricted oligoclonal bands (OCB) (data taken from the patient records), were considered as indicative of intrathecal synthesis of total IgG (as opposed to AQP4-specific IgG) [16]. The CSF/serum albumin ratio, Q_{Al} = Alb_{CSF}[mg/l]/Alb_{serum}[g/l], was used to assess the blood-CSF barrier function. The upper reference limit of Q_{Al} was calculated as 4 + (a/15) with a representing the patient’s age [19].

### Table 1 Epidemiological data and sample numbers

| Number of patients | Caucasian | Sex ratio, male:female | Relapsing course | Paired CSF/serum samples | Median age at LP (range) | Untreated at time of LP (%) |
|--------------------|-----------|------------------------|------------------|-------------------------|--------------------------|-----------------------------|
| Total              | 79        | 72/79 (91)             |                  |                         | 40 (15-72)               | 56/80 (70)                  |
| NMOSD              | 37        | 32/37 (87)             |                  |                         | 45 (11-72)               | 19/40 (48)†                 |
| Controls           | 42        | 40/42 (95)             |                  | 42                      | 39 (15-70)               | 37/40 (93)†‡                |
| MS                 | 28        | 26/28 (92)             |                  | 28                      | 38 (15-69)               | 2/27                        |
| OND                | 14        | 14/14 (100)            | 1:3.7            | 14                      | 45 (20-70)               | 1/13                        |

Diagnoses in the MS group included relapsing-remitting MS in 26; secondary progressive MS in 1; and primary progressive MS in 1. Diagnoses in the OND group included acute demyelinating encephalomyelitis in 4; non-longitudinally extensive transverse myelitis in 1; brain stem encephalitis of unknown aetiology in 1; autoimmune cerebellitis in 1; Herpes simplex virus encephalitis in 1; primary angitis of the CNS in 1; Behçet’s disease in 1; benign paroxysmal positional vertigo in 1; hydrocephalus arachnoiditis in 1; and spinal disc prolaps in 1. NMO was diagnosed according to reference [39]. LETM was defined as myelitis extending over three or more segments as demonstrated by magnetic resonance imaging. MS was diagnosed according to reference [40].† Treatments in the remaining cases included oral steroids, intravenous methylprednisolone, azathioprine, methotrexate, and cyclo-phosphamide; in 5 cases no exact data on the treatment status at time of LP were available.‡ In 2 cases, no exact data on the treatment status was available. LETM = longitudinally extensive transverse myelitis; MS = multiple sclerosis; NMO = neuromyelitis optica; n.d. = not determined; NMOSD = neuromyelitis spectrum disorders; ON = optic neuritis; OND = other neurological diseases.
Results

AQP4-Ab CSF status

AQP4-IgG was detected in 21/31 (68%) CSF samples from AQP4-Ab seropositive NMOSD patients. 0/14 CSF samples from AQP4-IgG seronegative NMOSD patients and 0/42 control samples were positive for AQP4-IgG. No significant difference regarding CSF AQP4-Ab frequency was found between acute relapses of ON (75%) and myelitis (59%). Detailed results are given in Table 2.

AQP4-Ab serum titres

Serum AQP4-IgG titres were determined in 26/31 NMOSD samples and were higher (median 1:1000; range, 1:250-1:12,500) in CSF AQP4-IgG-positive patients (n = 18) than in CSF AQP4-IgG-negative patients (1:250; 1:10-1:1000; n = 8) (p < 0.002; Mann-Whitney test). See Figure 1 and Table 2 for details.

Blood-CSF barrier function

Disturbances of the blood-CSF barrier as indicated by elevated QAlb were present in 11/18 (61%) AQP4-Ab serum and CSF positive NMOSD samples but only in 1/8 (13%) of the AQP4-Ab seropositive but CSF-negative NMOSD samples analysed (p = 0.036; Fisher exact test). In the AQP4-Ab seronegative group, blood-CSF barrier dysfunction was present in 6/9 (67%) cases. In the remaining cases not enough CSF or serum was available for QAlb determination. No significant correlation between AQP4-Ab titres and QAlb was found.

Total IgG in the CSF and serum

Evidence for intrathecal synthesis of total IgG as indicated by either CSF-restricted OCBs or elevated QIgG was present in 32.5% (13/40) NMOSD, and was slightly more frequent in AQP4-Ab seronegative NMOSD samples (50%, or 6/12) compared to seropositive samples (25%, or 7/28). No significant correlation with the AQP4-Ab CSF status was found; while 100% (7/7) of the samples with intrathecally produced total IgG that were obtained from AQP4-Ab seropositive patients were positive for CSF AQP4-Ab, also 57.1% (12/21) of the CSF samples from AQP4-Ab seropositive patients with

Table 2 Clinical findings, AQP4-Ab status in serum and CSF, and median AQP4-Ab serum titres in the various disease groups

| Diagnosis          | No of CSF samples | Acute attack at time of LP (%) | AQP4-Ab, serum (%) | AQP4-Ab, CSF (%) | Median serum titre (range; N) |
|--------------------|-------------------|--------------------------------|--------------------|-----------------|--------------------------------|
| NMOSD              | 45                | 29/40 (73)                     | 31/45 (69)         | 21/45 (47)      | 1000 (10-12500;26)             |
| NMO                | 26                | 16/22 (73)                     | 16/26 (62)         | 11/26 (42)      | 1000 (125-7000;12)             |
| LETM               | 8                 | 6/8 (75)                       | 7/8 (88)           | 5/8 (63)        | 250 (62.5-12500;7)             |
| ON                 | 11                | 7/10 (70)                      | 8/11 (73)          | 5/11 (45)       | 250 (10-7000;7)                |
| Relapse            | 29                | 29/29 (100)                    | 20/29 (69)         | 17/29 (59)      | 1000 (250-12500;20)            |
| Remission          | 11                | 0/11 (0)                       | 7/11 (64)          | 1/11 (9)        | 187.5 (10-250.6)               |
| AQP4-Ab seropositive | 31               | 20/31 (65)                     | 31/31 (100)        | 21/31 (68)      | 1000 (10-12500;26)             |
| AQP4-Ab seronegative | 14              | 9/14 (64)                      | 0/14 (0)           | 0/14 (0)        | Negative                       |
| Controls           | 42                | 31/38 (82)                     | 0/42 (0)           | 0/42 (0)        | Negative                       |
| MS                 | 28                | 22/28 (79)                     | 0/28 (0)           | 0/28 (0)        | Negative                       |
| OND                | 14                | 9/10 (90)                      | 0/14 (0)           | 0/14 (0)        | Negative                       |

AQP4-Ab = aquaporin-4 antibody; CSF = cerebrospinal fluid; LETM = longitudinally extensive transverse myelitis; MS = multiple sclerosis; NMOSD = NMO spectrum disorders; NMO = neuromyelitis optica; ON = optic neuritis; OND = other neurological diseases. § Not applicable in four patients with non-inflammatory neurological diseases.
neither OCBs nor QIgG elevation harboured AQP4-Ab (p = n.s.; Fisher exact test). Also, QIgG and AQP4-Ab titres showed no significant correlation.

**AQP4-Ab AI**

Intrathecal production (IP) of AQP4-IgG as defined by an AI > 4 was found in only 1/23 NMOSD samples analysed (4.3%) (AI_{AQP4} = 7). This patient had experienced a third attack of ON 12 days prior to LP (first LETM 10 months later) and had received high-dose methylprednisolone until nine days and low-dose steroids until five days prior to LP. The patient had co-existing acetylcholine receptor-antibody positive myasthenia gravis (treated with pyridostigmine) and thyroglobulin and thyroid peroxidase antibody positive thyroiditis. Clinical and laboratory findings at time of LP were otherwise unremarkable when compared to the remaining patients (Table 3). The sample was positive for OCBs, and QIgG (5.9; Qlim(IgG) = 5.3; intrathecal IgG fraction, 8.7%) and QAlb (7.2; age-adjusted upper reference limit = 5.5) were both elevated. A follow-up sample taken from the same patient one year later during another relapse of ON and still prior to initiation of long-term immunosuppression did not show evidence of AQP4-Ab IP anymore, though the antibody was still detectable in the CSF. In the remaining cases, not enough CSF and serum for QIgG and QAlb determination was available, so that AQP4-Ab could not be assessed (n = 5), or CSF titres that would indicate a positive AQP4-AI were below 1:1 (n = 3), so that testing was not possible (all of the latter samples were negative at 1:1 dilution).

**Impact of disease activity**

17/20 (85%) CSF samples from AQP4-Ab seropositive NMOSD patients obtained within 30 days after onset of relapse were AQP4-Ab positive but only 1/7 (14.3%) taken during remission (p = 0.0017; Fisher exact test, 2-tailed). Similarly, 17/18 (94.4%) samples positive for CSF and serum AQP4-Ab were taken during acute relapse; by contrast, only 3/9 (33.3%) AQP4-Ab CSF-negative but AQP4-Ab seropositive samples were obtained during relapse (p = 0.002; Fisher exact test). The median serum AQP4-Ab titre of those NMSOD samples taken during relapse (1:1000; range, 1:250-1:12500; n = 20) was higher than in NMSOD samples obtained during remission (1:187.5; 1:10-1:250; n = 6) (p = 0.0008; Mann-Whitney test). AQP4-Ab serum titres did not differ markedly between acute relapses of myelitis (median, 1:1000; n = 13) and ON (median, 1:2500; n = 6), and were relatively high in the only patient with acute relapse of simultaneous optic neuritis and myelitis (1:5000). Serum AQP4-Ab titre in the single patient with evidence for AQP4-Ab IP was 1:1000. IP of total IgG was found in 7/20 (35%) AQP4-Ab seropositive samples obtained during relapse but in 0/7 taken during remission. Disruption of the blood-CSF barrier was found both with AQP4-IgG seropositive samples taken during relapse (10/20; 50%) and with some of those obtained during remission (2/6; 33%). No significant differences between acute relapses of myelitis and acute relapses of ON were found regarding the frequency of total IgG IP or of blood-CSF barrier disruption.

**Impact of disease duration**

Median disease duration at time of LP was shorter (24.5 v 4.1 months) in the AQP4-Ab seronegative group (p = 0.056; Mann-Whitney test). Among seropositive patients, those positive for CSF AQP4-Ab had a longer disease duration (52.8 vs 19.45 months; p = 0.041). No correlation between serum titres and disease duration or time since relapse onset was found.

**Impact of treatment status**

Median serum AQP4-Ab titres did not differ significantly between untreated (n = 12; 1:625; range, 1:62.5-
1:12500) and treated (n = 14; 1:1000; 1:10-1:7000) NMOSD patients nor did the AQP4-IgG CSF positivity rate (64.3% and 75%, respectively). The only patient with positive AI_{AQP4} had received steroids until five days before LP.

**Longitudinal analysis**

In total, 8 follow-up samples from 8 patients with NMOSD were examined for AQP4-Ab (median latency, 381 days). No patient who was initially positive for serum AQP4-Ab was negative at follow up; the only patient negative at first testing was also negative at follow-up. However, the disease status (relapse or remission) at first and second LP was identical in all cases. Also, in all cases, CSF was positive for AQP4-Ab in both samples, if samples were taken during relapse, or negative, if samples were taken during remission; the only exception was one AQP4-Ab seropositive sample that was negative for CSF AQP4-Ab during a relapse of ON but positive during a relapse of myelitis.

**Discussion**

In this study we systematically evaluated the frequency of AQP4-IgG in the CSF and serum of Caucasian patients with NMOSD. Using a CBA employing recombinant human AQP4, we found CSF AQP4-IgG in ~70% of AQP4-IgG seropositive NMOSD samples, but in none of the MS or OND controls. AQP4-IgG CSF positivity was associated with higher AQP4-IgG serum titres and with dysfunction of the blood-CSF barrier. Moreover, AQP4-IgG was more frequently detectable during relapse (p = 0.0017). The latter finding most likely reflects an increase in serum AQP4-IgG titres during relapse, since no evidence of intrathecally produced AQP4-IgG was found in almost all cases analysed. Serum titres were indeed significantly higher during relapse than in remission in our patients (median, 1:1000 vs 1:187.5) (p = 0.001), which is in line with previous studies [13,20]. Interestingly, the cut-off serum AQP4-IgG titre that predicted CSF AQP4-IgG positivity (1:250) was similar to that found in Japanese patients with opticospinal MS [13].

Serum titres >1:250 were associated with acute disease in all cases, and titres ≤1:250 with remission. In contrast, no clear correlation was found in case the serum titre was 1:250. It should be mentioned as a caveat, however, that the number of samples obtained during remission was relatively low in this study (as LP is mainly done for acute disease). In a previous study that included samples obtained over a period of up to five years, we could demonstrate marked variations over time regarding AQP4-Ab concentrations during remission with no general cut-off for relapse induction (though relapses were always preceded by an relative increase in AQP4-Ab levels). The latter finding might indicate differences in AQP4-Ab affinity and specificity between patients and over time, but inter- and intraindividual variations regarding T-cell activation, cytokine levels, or BBB function may also play a role.

Serum AQP4-Ab titres did not differ markedly between untreated and treated NMOSD patients nor did the AQP4-IgG CSF positivity rate. This could be due to the fact that 11/14 samples from treated patients were taken during relapse, which was more commonly associated with high serum AQP4-Ab titres as well as with CSF AQP4-Ab.

Intrathecal AQP4-Ab production was present in only 1 out of 23 samples studied (4.3%). This sample was obtained during an acute relapse of ON. However, 20/23 AQP4-IgG CSF-positive samples with normal AI_{AQP4} values were also taken during relapse. AI_{AQP4} elevation seems thus not a suitable disease activity marker. The infrequency of AQP4-IgG IP suggests that in patients with NMOSD AQP4-Ab producing B cell clones usually reside in the systemic compartment. CSF AQP4-Ab might thus reflect passive diffusion of serum AQP4-Ab into the CSF. Accordingly, Takahashi et al. (2007), in a study on 12 Japanese patients, found that titres of CSF AQP4-IgG were almost proportional to serum AQP4-IgG in NMO, though, as a limitation, that study had not taken into account possible blood-CSF barrier disruption [13].

Although intrathecal AQP4-Ab production would then not be a prerequisite for inflammation in NMO, the single patient with a positive AI in our series (as well as a second recently published case with a slightly elevated AI[8]) indicates that intrathecal AQP4-Ab production can occur in NMOSD. However, the rarity with which it was detected argues against a major pathogenic function.

Unlike in MS, intrathecal total IgG synthesis in NMO does not persist over time [21-24]. It is of note that evidence for total IgG IP was present in 35% of seropositive samples obtained during relapse but in none of the seropositive samples obtained during remission. In line with this finding, Melanud et al., in a study so far only published as abstract, reported intrathecal total IgG synthesis in 45% of samples obtained in bout versus 0% in remission [24]. This might explain the low frequency of intrathecal total IgG production (17-33%) reported in the literature [1,21-23,25] compared to MS (95-100%). Similarly, AQP4-Ab might be present in the CSF only transiently as indicated by our finding of a higher AQP4-Ab CSF positivity rate during relapse. It is of note, however, that all eleven samples obtained at approximately (+/- 8 days) the same time from onset of relapse than the only AI positive sample (which was taken at day 12 after relapse onset), showed a negative
and human complement into the CSF indeed resulted in a model of NMO, injection of AQP4-Ab positive sera plasma and the fluids of the CNS [30,32,33]. In an animal model of NMO, injection of AQP4-Ab positive sera and human complement into the CSF indeed resulted in generation of NMO-like lesions in the absence of BBB disruption or pre-existing CNS inflammation [34]. In our study, seven patients had in fact normal QAB values but suffered from an acute attack, had high serum AQP4-Ab titres >= 1:250 (median, 1:1000), and were positive for CSF AQP4-Ab at time of LP. However, as a limitation, three of those patients were treated at time of LP, and in three the attack had started already 23, 28 and 30 days before LP. Moreover, QAin might not be sufficiently sensitive to reflect locally restricted BBB disruption, in particular in ON, which was present in 4 of the 7 patients at time of LP.

Finally, AQP4-Ab itself could induce BBB damage. Prolonged exposure to AQP4-Ab in the fenestrated perivascular and subpial spaces could lead to BBB disruption by gradual local inflammation or AQP4 internalisation, followed by exacerbation of the autoimmune response [30]. A number of recent in vitro and in vivo studies revealed that IgG from patients with NMO initiates endocytosis of AQP4, a process which alters the polarized expression pattern of AQP4 on the plasma membrane and, as a functional consequence, increases BBB permeability in vitro [35-37]. In AQP4 knock-out mice, the lack of AQP4 is mirrored by tight junction opening in brain microvessels, swelling of perivascular astrocytic processes, and BBB hyperpermeability [38]. Interestingly, disease activity was linked to higher CSF AQP4-Ab and to higher serum AQP4-Ab levels in this study and another,[13] indicating that CSF and/or serum AQP4-IgG exceeding a threshold value might be required to induce clinically relevant inflammation.

Conclusion
In summary, our study demonstrates that (1) AQP4-IgG is detectable in the CSF in most AQP4-IgG seropositive NMO patients but not in that of patients with MS or OND; (2) that the presence of CSF AQP4-IgG in patients with NMO is positively associated with acute disease relapse within 30 days prior of LP; AQP4-IgG serum titres >1:250; and with blood-CSF barrier disruption; but not with treatment status or the type of acute clinical disease (myelitis or ON) at time of LP; (3) a lack of quantitative evidence for intrathecal synthesis of AQP4-IgG in most NMO patients. Our findings argue against the need to test CSF for AQP4-Ab if the corresponding serum is negative for the antibody or AQP4-Ab serum titres are below 1:250. Moreover, our results suggest that intrathecal production of AQP4-Ab may not be a prerequisite of disease activity in NMO.

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Authors’ contributions
SJ conceived and designed the study. SJ, CP, and KPW were involved in carrying out the immunassays. SJ, DF, FP, KR, RB, PR, RR, WK, KW, KPW, and BW participated in CSF and data collection. SJ performed the statistical analysis and wrote the initial draft. SJ, DF, FP, KR, RB, PR, RR, WK, KPW, and BW participated in the preparation of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests
SJ, DF, FP, KR, RB, PR, RR, WK, and BW declare no competing interests. The cells used in this study were kindly provided by Euroimmun, Luebeck, Germany. KPW and CP are employees of Euroimmun. Euroimmun had no role in study design, data collection or analysis, preparation of the manuscript, or decision to publish.

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