debate. The recent discovery of a specific IgG against NMO, designated NMO-IgG, suggests that NMO is a distinct disease entity with a fundamentally different etiology from MS [3,4]. Because NMO-IgG has been reported to be present in about 50–60% of OSMS patients [3,5], OSMS in Asians has been suggested to be the same entity as NMO. However, the observations that NMO-IgG is not found in all cases of NMO or OSMS [5–7], and that 5–10% of classical MS patients also carry the antibody [3,5,7] cast doubt on the simple dichotomy of categorizing human demyelinating disease into MS and NMO. In Asians, the mechanism underlying the formation of LESCLs is heterogeneous, and the disease condition in those with NMO-IgG does not completely overlap with OSMS in Asians [6,7]. In this review, possible mechanisms underlying NMO with NMO-IgG and OSMS without the antibody are discussed.

## 2. History and nosological problems of neuromyelitis optica

The nosological position of NMO has been a matter of debate since Dević first summarized cases with optic neuritis and spinal cord disease [8]. Originally, NMO was considered to be a monophasic disease that simultaneously affected both the optic nerves and spinal cord. However, several reported cases showed a relapsing course [8–11] and, later, Wingerchuk et al. [2] proposed a nosological entity of relapsing NMO and described its diagnostic criteria. Some cases of relapsing NMO even showed general and local cerebral symptoms, such as Jacksonian seizure, headache, vomiting and dysarthria [8–11]. As well, pathologically, small foci of demyelinating plaques are frequently observed in postmortem brains from NMO patients [10,11]. The occurrence of relapse and brain symptoms, and the pathologically demonstrated presence of additional demyelinating plaques, indicate the existence of considerable overlap between MS and NMO. This situation imposes difficulty for differentiating NMO from MS clinically and pathologically.

## 3. History and features of MS in Asians

Before the late 1950s, MS was rarely reported in Asia countries. In 1958, Okinaka et al. [12] reported the clinical features of 270 cases of MS and allied disorders that had been diagnosed between 1890 and 1955. In this series, 65% had NMO, 24% had MS and 2% had Schilder's disease, while the other cases had unclassifiable diseases. Among the NMO cases, 48% showed a relapsing course and the authors found many intermediate cases between MS and NMO. Thereafter, in Japan and the rest of Asia, NMO has been used to describe monophasic cases showing bilateral optic neuritis and transverse myelitis within an interval of less than several weeks, and relapsing cases have usually been classified as MS.

A comparative study of MS between Japanese and British patients done by Shibasaki et al. [13] disclosed the characteristic features of MS in Asians. These included selective and severe involvement of the optic nerves and spinal cord, rapid progression, infrequent secondary progression, rare familial occurrence, and no association of MS patients as a group with any HLA allele. MS in Asians was thus considered to be modified from that seen in Western populations as a whole.

In 1996, Kira et al. [14] first reported different features between opticospinal (OSMS, Asian type MS) and conventional MS (CMS, Western type MS) and proposed clinical classification criteria for OSMS, namely, selective involvement of the optic nerves and spinal cord by clinical symptomatology with or without minor brainstem signs. Thereafter, phenotypic classification and characterization have been actively undertaken by Japanese researchers. These studies have revealed that 15–40% of MS cases in Japan are of an OSMS phenotype and have clarified the demographic features of OSMS [1]. Compared with CMS, OSMS has the following characteristic features in Asians: (1) higher age at onset; (2) female preponderance; (3) frequent relapses; (4) greater disability due to severe optic nerve and spinal cord damage; (5) fewer brain MRI lesions; (6) LESCLs extending over many vertebral segments on spinal cord MRI; (7) marked pleocytosis and neutrophilia in cerebrospinal fluid (CSF); and (8) absence of oligoclonal bands (OB) in CSF. Moreover, HLA association is also distinct between the two subtypes: *HLA-DRB1*1501* is associated with the CMS phenotype [14], as seen in Caucasian patients with MS, while *HLA-DPB1*0501* is associated with OSMS in Japanese [15]. On the other hand, CMS patients show similar features to MS in Westerners, including the same *HLA-DRB1*1501* association [1]. These observations suggest that the two subtypes have distinct mechanisms; however, there remains considerable overlap between the two disease entities, primarily because of the arbitrariness and ambiguity encompassed by the clinical findings-based classifications.

## 4. Discovery of NMO-IgG and its relevant antigen in NMO

NMO-IgG was originally found in 73% of NMO patients without brain lesions on MRI by immunohistochemical staining of mouse cerebellar tissue sections [3]. Later, the relevant antigen recognized by the antibodies was found to be aquaporin-4 (AQP4) [4]. Thereafter, AQP4- or GFP–AQP4 fusion protein-transfected cells have been used for immunostaining. AQP4 is one of the major water channel proteins in the CNS and is abundantly expressed throughout the CNS including the cerebrum and cerebellum. Although NMO mainly affects the optic nerves and spinal cord, Pittock et al. [16] reported that asymptomatic brain lesions on MRI are common in NMO patients with anti-AQP4 antibody. According to the revised criteria for NMO (Table 1), even the presence of symptomatic brain lesions does not exclude a

Table 1
The revised criteria for neuromyelitis optica.

| |
|---|
| Definite NMO |
| 1. Optic neuritis |
| 2. Acute myelitis |
| 3. At least 2 of 3 supportive criteria |
| 1. LESCL |
| 2. Brain MRI not meeting Dx. criteria for MS |
| 3. NMO-IgG (+) |

Wingerchuk et al. (2006) (Ref. [17]).

diagnosis of NMO based on the presence of NMO-IgG [17]. According to Pittock et al. [18], brain lesions in NMO are preferentially observed in regions where AQP4 is abundantly present, such as the bilateral diencephalic regions adjacent to the third ventricles, the pontine tegmentum and cerebellum surrounding the fourth ventricles, and the periventricular white matter adjacent to the lateral ventricles.

## 5. Positivity rates of NMO-IgG and anti-aquaporin-4 antibody in various ethnic groups

### 5.1. Seroprevalence and sensitivity

One of the confounding problems concerning the involvement of NMO-IgG in the diagnosis of NMO is that NMO-IgG is not detected in all NMO patients (Table 2). In Caucasians, 73% were positive in Lennon's original report [3] and similar figures have also been reported elsewhere: 22/36 (61.1%) in Jarius et al. [19] and 21/37 (56.72%) in Paul et al. [20] were positive. Recently, Fazio et al. [21] conducted immunofluorescence, flow cytometry and radioimmunoprecipitation assays in Italian patients with NMO and found 30–47% were positive. In Africans and their descendants, much lower positivity rates were reported: 33.3% in Caribbean patients with NMO [22] and 5% in African–American patients with OSMS [23].

In Japanese, Nakashima et al. [5] reported detection of NMO-IgG in 63% of OSMS patients and 15% of CMS patients. Recently, the same group also reported that 20 of 22 NMO patients had the anti-AQP4 antibody, while none of the 53 MS patients did (90% versus 0%) [24]. In their series, all 22 NMO patients, all female, were defined as cases fulfilling all items of the 2006 NMO criteria [17] except for NMO-IgG-seropositive status. From reports before the discovery of NMO-IgG, the male to female ratio in relapsing NMO was 1:5 at most and 1:1 in the monophasic type [2]. Therefore, considering the extremely high female ratio in Takahashi's series [24], there appears to have been an obvious subject bias in their study. Tanaka et al. [25], in their selected series of MS patients, independently reported that anti-AQP4 antibody positivity rate was 16/26 (61.5%) in OSMS patients with LESCLs and 0/21 (0%) in CMS patients without LESCLs.

Table 2
Positivity rates for NMO-IgG/anti-AQP4 antibody among races.

| Race | Disease | NMO-IgG/anti-AQP4 antibody (%) |
|---|---|---|
| Caucasians [3,19–21] | NMO | 30–73* |
| Northern Japanese [5] | OSMS | 63* |
| Southern Japanese [6] | OSMS | 27* |
| Caribbean [22] | NMO | 33 |
| Indian [29] | NMO spectrum disorder | 5* |
| African–American [23] | OSMS | 5 |

* Measured by Mayo Clinic.

It is critical that anti-AQP4 antibody be examined in a blind fashion in a large number of consecutive MS patients covering the whole spectrum of MS and that the positivity rate be compared with that in NMO-IgG patients. We undertook such a study using serum samples with NMO-IgG status predetermined at the Mayo Clinic; we found that their anti-AQP4 antibody assay was 83.3% sensitive and 100% specific for NMO-IgG [6]. According to the results using this assay system, the anti-AQP4 antibody was positive in 27.1% (13/48) of OSMS patients, 5.6% (3/54) of CMS patients, 0% (0/52) of those with other neurological diseases, and 0% (0/35) of healthy controls [6]. Among the OSMS patients, the antibody positivity rate was highest (55.6%) in OSMS patients with both LESCLs and MS-like brain lesions fulfilling the Barkhof criteria for MS [26], although NMO-IgG was originally described in patients with exclusively optic nerve and spinal cord lesions.

There are obvious discrepancies in the detection rates among the above-mentioned series in Japanese. The reasons for these may relate to differences in the subjects used: NMO versus OSMS patients with LESCLs; selected versus consecutive patients; northern versus southern Japanese patients that have been shown to have somewhat distinctive features in clinical phenotype by a recent nationwide survey [27,28]. They could also relate to the methods used: AQP4-transfected versus GFP–AQP4 fusion protein-transfected; fixed transfected cell specimens versus unfixed ones; 1:4 dilution versus 1:400 dilution. However, even in the studies done by the Mayo clinic, there are considerable differences in positivity rate, indicating that the difference is in part attributable to differences between subjects (Table 2) [3,5,6,19–23,29]. It remains to be elucidated whether the 30–70% of NMO patients who fulfilled the NMO diagnostic criteria and did not carry the antibodies are truly seronegative NMO patients or false negatives due to the low sensitivity of the assay.

### 5.2. Specificity

NMO-IgG has not been described in other inflammatory diseases in Westerners; however, 9% of MS cases in Lennon's

original series did have the antibodies [3]. To date, 5–15% of tested MS cases were found to be positive for NMO-IgG or anti-AQP4 antibody [3,5–7,20]. Even in the above-mentioned report by Pittock et al. [16], describing the occurrence of brain lesions in NMO patients, 10% of NMO-IgG-positive patients had brain lesions that were indistinguishable from MS lesions. This indicates the existence of considerable overlap between NMO and MS, which cannot be ignored.

## 6. Epitopes and titers of anti-AQP4 antibody

### 6.1. Epitopes

The finding that positive sera stained the cell surfaces of AQP4-transfected cells, but not the cytoplasm, suggests that patients' sera recognized the conformational epitopes of the molecule expressed on the cell surface [4,6]. Recently, by comparing the reactivity of NMO patients' sera against human, mouse and rat AQP4 proteins, which have several amino acid substitutions, the third extracellular loop of AQP4 was suggested to be the major epitope for AQP4 antibody in NMO patients [30]. AQP4 has two isoforms: the longer M1 isoform and the shorter M23 isoform lacking the N-terminal 22 amino acids. Only the presence of the M23 isoform induces formation of an orthogonal array of particles (OAPs) [31] and Nicchia et al. [32] reported that the NMO-IgG epitope is intrinsic in AQP4 assemblies into OAPs. All of the immunofluorescence studies mentioned above used the M1 isoform; however, in cells transfected with the M1 coding sequence, the M23 isoform was also detected because of leaky scanning for synthesis of the shorter M23 isoform from the second methionine [33].

### 6.2. Titers and seroconversion

Takahashi et al. [24] claimed that anti-AQP4 antibody titers showed a strong positive correlation with the spinal cord lesion length ($R = 0.9108$), while others have not found any correlation between the two parameters [6,7,34,35]. Although the NMO-IgG/anti-AQP4 antibody usually appears in the early course of the disease [36], seroconversion of NMO-IgG/anti-AQP4 antibody during the course of illness is observed in some patients [6,7]. This may indicate the possibility that the antibody is produced secondarily following tissue destruction in some patients, as seen in MS patients in whom various autoantibodies emerge during the clinical course; some of them target even neural antigens and are shown to be functional *in vivo* [37]. A recent report has indicated the emergence in animals with myelin-oligodendrocyte glycoprotein-induced EAE of anti-AQP4 antibody [38]. Thus, it will be crucial to examine whether antibodies recognizing conformational epitopes can be secondarily induced in myelin-sensitized EAE animals. If so, then it will be necessary to test whether such antibodies can modify the clinical course.

## 7. Clinical and neuroimaging characteristics of anti-AQP4 antibody-positive NMO patients and anti-AQP4 antibody-negative OSMS patients in Asians

Anti-AQP4 antibody-positive NMO patients demonstrate a higher age at onset, marked female preponderance (the male:female ratio is around 1:10), high frequency of relapses, severe visual disturbance due to severe optic nerve, high frequency of acute transverse myelitis (ATM) due to severe spinal cord damage, rare occurrence of secondary progression, LESCLs on spinal cord MRI, marked pleocytosis and neutrophilia in cerebrospinal fluid (CSF), and absence of oligoclonal bands (OB) in CSF, as compared with classical MS patients [5–7,17]. However, most conditions are also common to anti-AQP4 antibody-negative OSMS patients [6,7].

In Western MS series, spinal cord lesions usually span less than two vertebral segments and occupy less than one-half of a spinal cross-section, preferentially involving the peripheral white matter [39]. LESCLs extending over three vertebral segments are rarely seen in classical MS patients in Western populations; 3% according to Tartaglino [39]. However, in a recent study on Western populations, Bot et al. [40] reported a relatively high frequency (12.5%) of LESCLs in Western MS patients; 12.5% had long spinal cord lesions. On the other hand, in Asians, LESCLs are frequently observed in not only OSMS patients, but also CMS patients [41–44]. Indeed, LESCLs are seen in about half of OSMS cases and a quarter of CMS cases, reflecting the severe spinal cord damage seen in Asian MS patients. Detailed analyses of LESCLs on MRI disclosed that LESCLs in anti-AQP4 antibody-positive patients were located in the upper to middle thoracic cord, while those in anti-AQP4 antibody-negative OSMS patients were present throughout the cervical to thoracic cord [6]. In axial planes, the former most frequently involved the central gray matter while the latter showed a holocord involvement pattern [6]. By contrast, in anti-AQP4 antibody-negative CMS patients, both short and long spinal cord lesions preferentially involved the mid-cervical cord, presenting a peripheral white matter-predominant pattern [6].

Unexpectedly, anti-AQP4 antibody-positive NMO patients had a greater frequency of brain lesions than anti-AQP4 antibody-negative OSMS patients with LESCLs, further suggesting that the conditions are distinct [6,7]. Moreover, anti-AQP4 antibody-positive NMO patients showed less frequent responses to interferon beta (IFNβ)-1b than anti-AQP4 antibody-negative OSMS patients with LESCLs [6].

In our series, multiple logistic analyses disclosed that the emergence of the anti-AQP4 antibody was positively associated with only a higher relapse rate, but not LESCLs [6]. These observations collectively suggest that LESCLs are distinct according to anti-AQP4 antibody status and clinical phenotype, and that the mechanisms producing LESCLs are heterogeneous, even in cases with optic-spinal presentation,

namely AQP4 autoimmunity-related and -unrelated. In a randomized double-blind study of the efficacy of IFNβ-1b in Japanese patients with MS, the drug was found to be equally effective in CMS and OSMS patients [45]. Responsiveness to IFNβ-1b in OSMS patients is well explained by the presence of anti-AQP4 antibody-negative OSMS patients who can respond to the drug, suggesting the possibility that anti-AQP4 antibody-negative OSMS constitutes a spectrum of MS.

## 8. The nature of brain lesions in anti-AQP4 antibody-positive patients as determined by neuroimaging

Anti-AQP4 antibody-positive NMO patients occasionally develop huge brain lesions. Such extensive white matter lesions in anti-AQP4 antibody-positive NMO patients demonstrate high signal intensity on ADC maps and low or isointensity on diffusion-weighted MRI images (DWI), suggesting the nature of the lesions to be vasogenic edema [6,46]. On magnetic resonance spectroscopy (MRS), a high choline peak and a low n-acetyl aspartate (NAA) peak are observed, compatible with acute demyelination [6,46]. These findings strongly suggest that the nature of the lesions in anti-AQP4 antibody-positive MS patients is vasogenic edema. The frequent occurrence of spinal cord edema in the acute phase and its resolution in the convalescence phase following methylprednisolone pulse therapy in anti-AQP4 antibody-positive MS patients is also consistent with vasogenic edema. However, interestingly, even in such extensive brain lesions, gadolinium enhancement of the lesions is absent or scant [46,47], except for cases complicated with other systemic autoimmune diseases, suggesting preserved integrity of the blood–brain barrier (BBB) in this condition. Anti-AQP4 antibody may disturb AQP4 water channel function, thereby leading to inappropriate water transfer in the presence of intact BBB. Contrarily, Ito et al. [48] recently reported that multiple patchy enhancing lesions with blurred margins, described as "cloud-like enhancement", are found in 90% of NMO patients with contrast enhancement, suggesting breakdown of BBB in this condition. Therefore, the mechanism underlying relapse could be heterogeneous, even among individuals with anti-AQP4 antibody.

## 9. Background for NMO-IgG/anti-AQP4 antibody production

### 9.1. Autoimmune background

Relapsing NMO with anti-AQP4 antibody is frequently associated with other autoantibodies and autoimmune diseases, such as Sjögren syndrome, systemic lupus erythematosus, autoimmune thyroiditis, and myasthenia gravis, in Westerners [2,49]. Although in Asian OSMS patients such a high frequency of coexistent autoimmune disease has not been reported [1], other autoantibodies, such as SSA and SSB, as well as other autoimmune diseases, such as Sjögren syndrome, are frequently present even in Asian patients with anti-AQP4 antibodies [6,7,50–52]. Therefore, an autoimmune-prone background, especially heightened humoral autoimmunity, seems to be an important factor in the production of the anti-AQP4 antibody. We found that, among anti-AQP4 antibody-positive individuals, Th1 cell percentage showed a significant negative correlation with anti-AQP4 antibody titer, and that those with SSA/SSB antibody had significantly higher titers of anti-AQP4 antibody [7]. Therefore, high titer anti-AQP4 antibody seems to be produced in those with a heightened humoral autoimmune background, a Th2-prone condition. Considering that OSMS patients with low titer anti-AQP4 antibody showed similar clinical and immunological features to those of OSMS patients without the antibody, it may be possible that low titer anti-AQP4 antibody is secondary to severe tissue destruction [7].

### 9.2. HLA

In our series, the frequency of *HLA-DPB1*0501* was significantly increased in anti-AQP4 antibody-positive patients as compared with healthy controls, but not in anti-AQP4 antibody-negative OSMS patients with LESCLs [53]. In Caucasians, HLA-DRB3 was reported to be over-represented in NMO patients [54]. The HLA-DRB1*15 allele, which is the strongest disease susceptibility allele for MS in Westerners, is under-represented in NMO patients [23,54]. More recently, Isobe et al. [55] studied the epistatic interactions of HLA-DRB1 alleles in Japanese patients with MS and NMO; the frequency of HLA-DRB1*09 was decreased in both anti-AQP4 antibody-negative MS and anti-AQP4 antibody-positive patients with NMO spectrum disorders, while HLA-DRB1*12 increased the risk of anti-AQP4 antibody-positive NMO spectrum disorders. HLA-DRB1*09/15 decreased the risk of MS, whereas HLA-DRB1*12/15 increased the risk of NMO. These findings suggest the possibility that the anti-AQP4 antibody and NMO are induced with a certain genetic background; however, the genes associated with anti-AQP4 antibody production and NMO susceptibility could vary from race to race.

### 9.3. Paraneoplastic condition

NMO-IgG/anti-AQP4 antibody has been found in patients with malignancies, such as breast cancer, lung cancer, uterus cancer, thymoma, B cell lymphoma. Some showed NMO features while others had no NMO symptoms [56]. There are several reports describing NMO cases whose sera harbored anti-AQP4 antibody long before the onset of NMO [56,57]. AQP4 has now been added to the long list of onco-neural antigens. The occurrence of a healthy carrier state contradicts the primary proposed role of the anti-AQP4 antibody.

### 9.4. Infections

Wingerchuk et al. [2] reported that monophasic NMO is associated with preceding infection while relapsing NMO is associated with other autoimmunity. It was recently reported that 88% of parainfectious NMO is monophasic [58]. Hypercomplementemia and elevation of C-reactive protein are seen in anti-AQP4 antibody-positive patients with NMO spectrum disorders at relapse; however, such systemic inflammatory reaction is rare in classical MS [59]. Considering its relapsing nature, specific acute infection is less likely to play a role in causing relapsing NMO with anti-AQP4 antibody. However, we found that *Helicobacter pylori* infection is more frequent in anti-AQP4 antibody-positive individuals than anti-AQP4 antibody-negative CMS patients and healthy controls [60]. Chronic persistent infection may in part contribute to the development of NMO through molecular mimicry between bacterial AQP and human AQP4, and the effects of the infectious agents' products rendering the BBB leaky.

## 10. Immunohistopathological studies on NMO and Asian OSMS

In NMO, intense demyelination, a great loss of axons, perivascular lymphocytic infiltration, microglial proliferation and vascular proliferation are seen in optic nerve and spinal cord lesions; these can occasionally lead to cystic cavities in severely involved areas [9–11]. Astrocytosis is scarce in some necrotic lesions but considerable in others. On the other hand, the neuropathological features of MS in Asians are as follows: (1) preferential occurrence of lesions in the optic nerves and spinal cord; (2) necrotizing lesions with occasional cavity formation not only in the spinal cord and optic nerves but also in the cerebrum; (3) poor gliosis; and (4) poor perivascular cuffing in the necrotic form [12,61–63]. Perivascular cuffing and gliosis varied regionally. Spinal cord lesions were usually most severe in the lower cervical to the mid thoracic cord. Polymorphonuclear leukocyte infiltration was occasionally seen in severe lesions in Asian MS patients, but eosinophil infiltration, as described in Western NMO patients [64], was not reported in early [12,61–63] or more recent literature [65].

Ikuta et al. [63] compared MS pathology between 70 American and 75 Japanese autopsy cases and found that 47% of Japanese cases showed selective involvement of the optic nerves and spinal cord, while 13% of American cases also showed limited involvement of the optic nerves and spinal cord. Considering all of the reported evidence, it appears appropriate to assume that MS and NMO are not easily separable based on pathological findings alone.

More recently, Lucchinetti et al. [64] described perivascular immune complex deposition (IgM, IgG and C9neo) in a rim or rosette pattern. A similar finding has been reported by a Japanese group [66]. Misu et al. [66] reported extensive loss of AQP4 accompanied by decreased GFAP staining in active perivascular lesions where MBP staining was relatively preserved in postmortem Japanese NMO cases. Loss of AQP4 with MBP preservation was observed in 18 of 22 active inflammatory lesions, 11 of 25 active demyelinating lesions and 3 of 8 chronic active lesions, while it was not apparent in 12 chronic inactive lesions. Instead, losses of both AQP4 and MBP were found in 4 of 22 active inflammatory lesions, 13 of 25 active demyelinating lesions, 4 of 8 chronic active lesions and 7 of 12 chronic lesions. MBP loss with an AQP4 preservation pattern was seen in none of 22 active inflammatory lesions, 1 of 25 active demyelinating lesions, 1 of 8 chronic active lesions and 3 of 12 chronic inactive lesions. By contrast, in MS plaques, AQP4 was never lost but rather was upregulated, reflecting astrogliosis. Based on the presence of immunoglobulin and complement deposition in active perivascular lesions, Misu et al. [66] postulated that astrocytic impairment associated with the loss of AQP4 by humoral immunity is the primary event in NMO, suggesting a primary role for the anti-AQP4 antibody in NMO pathology. Roemer et al. [67] made similar observations regarding novel NMO lesions in the spinal cord and medullary tegmentum extending to the area postrema where the blood–brain barrier is absent.

However, even in Roemer's report [67], some MS plaques showed selective AQP4 loss. We [68] also found that, in some MS lesions, AQP4 was lost extensively far beyond the areas of myelin loss. Kobayashi et al. [69] reported an autopsied case of NMO showing preservation of AQP4 in the severe lesions in the spinal cord and medulla, and in the demyelinating lesions in the optic nerve. By pathological study of 11 autopsied NMO and NMO spectrum disorder cases, we also found that some demonstrated selective AQP4 loss while others showed preservation of AQP4, even in the acute lesions, and that even in identical individuals some lesions showed AQP4 loss while other lesions showed up-regulation of AQP4 [68]. Therefore, AQP4 down-modulation does not seem to be specific for NMO, and the mechanisms underlying AQP4 down-modulation could be heterogeneous.

## 11. Pathogenicity of NMO-IgG/anti-AQP4 antibody *in vitro* and *in vivo*

Sera and IgG from NMO patients with NMO-IgG/anti-AQP4 antibody induce astrocyte damage and death in primary culture only in the presence of complements [70–72], while in the absence of complement they do not affect AQP4 water channel function in astrocytes [32]. IgG containing anti-AQP4 antibody from NMO-IgG-seropositive NMO patients reproduces astrocyte loss *in vivo* only when myelin basic protein (MBP)-specific T cells are transferred to cause experimental autoimmune encephalomyelitis (EAE) [73–75]. However, when AQP4 antibody was injected into young rats with a leaky BBB, or after transfer of non-encephalitogenic T cells, it did not induce any disease or neuropathological alterations in the CNS [75].

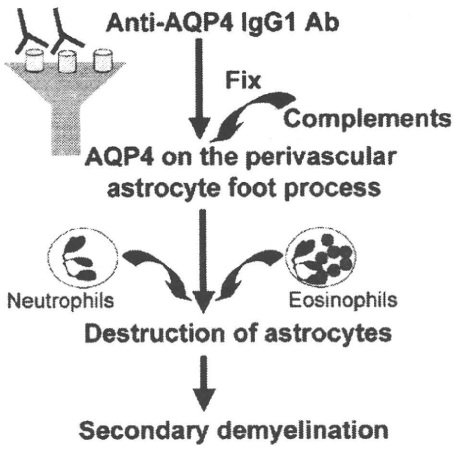


Fig. 1. Destruction of an astrocyte foot process by complement-activating anti-aquaporin-4 (AQP4) antibody. Once the anti-AQP4 IgG1 antibody crosses the blood–brain barrier, it binds to AQP4 on the astrocyte foot process, and fixes and activates complements. Activated complements mobilize neutrophils and eosinophils, which then produce severe tissue damage, and cause secondary demyelination. Disruption of the astrocyte foot process prolongs resolution from vasogenic edema caused by inflammation.

## 12. Proposed mechanism of NMO based on anti-AQP4 autoimmunity

Based on the high specificity of anti-AQP4 antibody and the selective loss of AQP4 in NMO lesions, it is postulated that the complement-activating anti-AQP4 antibody plays a pivotal role in the development of NMO lesions [76]. Once anti-AQP4 antibody gets across the BBB, it binds to AQP4 molecules on the astrocyte foot processes and activates complements (Fig. 1). Activated complements mobilize neutrophils and eosinophils that then facilitate tissue destruction. The observation that the anti-AQP4 antibodies so far examined are all IgG1 subclass [7] that can efficiently fix complements is compatible with such a hypothesis. The *in vitro* and *in vivo* pathogenic effects of anti-AQP4 antibody further support this notion. Here, demyelination is secondary to destruction of astrocytes, which is supposed to be fundamentally distinct from the primary demyelinating mechanism executed by myelin antigen-specific T cells and anti-myelin autoantibodies.

## 13. Concerns about the proposed mechanism of NMO based on anti-AQP4 antibody

There are several concerns surrounding the above-mentioned hypothesis based on anti-AQP4 antibody. First, in the presence of high titers of anti-AQP4 antibodies, some patients remained in remission [7], and there are cases who carry anti-AQP4 antibody without showing NMO presentation [56]. Because AQP4 is present in the astrocyte foot processes behind the BBB, additional factors that disrupt the BBB and render the antibody able to enter the CNS across the BBB may be necessary to induce relapse. In addition, the fact that anti-AQP4 antibody titers [6,7,34,35] appear to have no correlation with clinical parameters in most studies to date further support the prerequisite for some additional factor to induce relapse. Indeed, in animal models, there is a requirement for myelin antigen-specific T cells for anti-AQP4 antibody to operate *in vivo* in the CNS [74,75]. We found that in OSMS and NMO patients' peripheral blood T cells reactive to myelin antigens, such as MBP, proteolipid protein, and myelin-oligodendrocyte glycoprotein, showed intra- and inter-molecular epitope spreading [76], suggesting that T cells are already stimulated with myelin antigens *in vivo* in these patients. Second, AQP4 is present in retina, distal collecting tubules, gastric mucosa, muscle and lung, and NMO-IgG binds to these structures [4,77]; however, no impairments in these organs have been observed to date. In particular, although Müller cells, which are equivalent to astrocytes, abundantly express AQP4 in the foot process adjacent to the blood vessels in retina, no severe inflammation has ever been reported in anti-AQP4 antibody-positive NMO patients, suggesting that the presence of complement-fixing anti-AQP4 antibody is not enough to produce tissue damage. Moreover, AQP4 expression is ubiquitous throughout the CNS, although its expression level varies, being high in the gray matter of the spinal cord [66]. Cerebral gray matter and cerebellum abundantly express AQP4; however, these sites are seldom involved in NMO. Such a ubiquitous presence of AQP4 cannot explain the selectiveness of lesion distribution, namely in the optic nerves and spinal cord. Third, the deposited immunoglobulins in postmortem NMO lesions are mainly IgM [64] while the anti-AQP4 antibodies described are all IgG. We observed that some NMO lesions show perivascular deposition of complements and IgG in acute lesions, while no AQP4 loss is found (submitted for publication) [68]. Thus, perivascular complement and IgG deposition does not strictly correlate with AQP4 loss. Finally, AQP4 loss has been observed in MS plaques by researchers at several independent institutions, while preservation of AQP4 in NMO lesions is also seen by several groups [68,69]. These observations suggest that AQP4 loss is not completely linked to NMO lesion formation, and that there are two subtypes of NMO/OSMS with LESCLs, at least in Asians (Fig. 2).

## 14. Alternative mechanisms of OSMS in Asians based on CSF and peripheral blood cytokine/chemokine profiles

In peripheral blood, OSMS shows a pronounced T-helper-1 (Th1) and T-cytotoxic-1 (Tc1) shift, where IFNγ-producing T cells predominate over IL-4-producing T cells throughout the relapse and remission phases [78,79]. We previously reported that IL-17 is upregulated in the CSF of OSMS patients and that levels of both IL-17 and the downstream cytokine IL-8 in CSF show a significant positive correlation

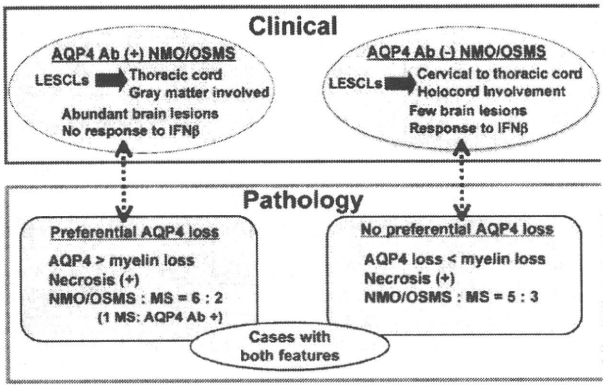


Fig. 2. Two subtypes of NMO/OSMS with LESCLs in Asians. There are NMO-IgG-seropositive and -seronegative cases with NMO/OSMS, which show some difference in clinical features. Pathologically, there are also two subtypes of NMO: one presenting with preferential AQP4 loss and one that does not. Even in MS plaques, AQP4 loss is occasionally seen. Some cases show both features in different lesions. Ab: antibody; AQP4: aquaporin-4; LESCLs: longitudinally extensive spinal cord lesions; MS: multiple sclerosis; NMO: neuromyelitis optica; OSMS: opticospinal multiple sclerosis.

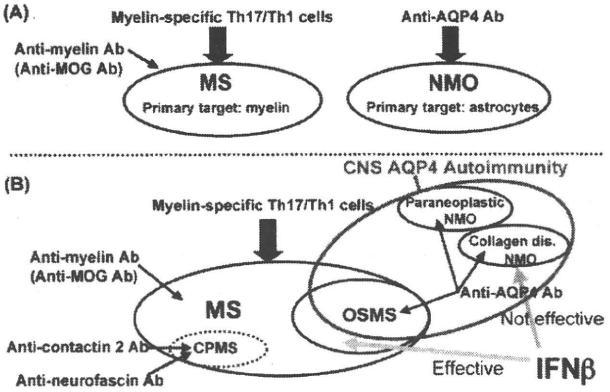


Fig. 3. Two hypothetical mechanisms of MS and NMO. In (A), myelin is a primary target of T cells and antibodies in MS, whereas in NMO, astrocytes are a primary target of anti-AQP4 antibody. In (B), myelin-specific T cells initiate CNS inflammation and antibodies recognizing various components of CNS antigens modify the clinicopathological features. Ab: antibody; AQP4: aquaporin-4; CPMS: chronic progressive multiple sclerosis; MOG: myelin-oligodendrocyte glycoprotein; MS: multiple sclerosis; NMO: neuromyelitis optica; OSMS: opticospinal multiple sclerosis.

with spinal cord lesion length [65]. Recently, by simultaneously measuring the levels of 27 cytokines and chemokines in CSF from patients with various causes of myelitis, we found that IL-17, IFNγ, and G-CSF were specifically elevated in OSMS patients, irrespective of the presence or absence of anti-AQP4 antibody [80]. IL-17 (IL-17A) is exclusively produced by Th17 cells, which are CD4+ T cells recently shown to be a distinct lineage from Th1 and Th2 cells [81]. Increasing evidence suggests that Th17 cells, but not Th1 cells, are responsible for organ-specific autoimmune diseases, such as EAE [81,82]. IL-8 is a chemokine for neutrophils. In OSMS patients, CSF neutrophilia and infiltration of neutrophils to severe lesions are characteristic [65]. Hence, elevated IL-8 may be partly responsible for such neutrophil activation and mobilization in OSMS. Indeed, the level of myeloperoxidase, an activated neutrophil product, is increased in sera from OSMS patients, especially in those with LESCLs at relapse [42]. Th17 cells carrying granzyme B have recently been shown to efficiently disrupt BBB tight junctions and loosen the BBB [83]. Therefore, autoimmune Th17 cells may initiate BBB disruption and inflammation in OSMS (Fig. 3), causing vasogenic edema in the CNS, regardless of the anti-AQP4 antibody status. After myelin-specific T cells initiate CNS inflammation, antibodies recognizing various components of CNS antigens might modify the clinicopathological features of MS. In such a scenario, NMO without overt autoimmune diseases or paraneoplastic conditions may represent one extreme end of an MS spectrum.

Regarding other factors with possible effects on vascular permeability, we previously reported that the levels of vascular endothelial growth factor (VEGF) in sera were significantly elevated in OSMS patients, showing a significant positive correlation with spinal cord lesion length [43]. IL-17 has also been shown to induce VEGF production in target tissues [84]. Moreover, a mutation in the *platelet activating factor* (*PAF*) *acetylhydrolase* (*PAF-AH*) gene, which inactivates its enzymatic activity (required to metabolize PAF into an inactive form), is significantly more frequent in OSMS patients than healthy controls [85]. Indeed, PAF-AH activity in peripheral blood is decreased in OSMS patients [85]. This likely prolongs PAF activity and increases vascular permeability as well as vascular growth. These vascular-acting factors may also enhance tissue edema in OSMS.

Given that AQP4 knockout mice showed prolongation of vasogenic edema [86], but a decrease in the level of cytotoxic edema [87], anti-AQP4 antibody produced either by a heightened humoral autoimmune background or secondarily by tissue breakdown may prolong resolution of tissue edema, thereby contributing to further tissue destruction in NMO and OSMS patients. NMO-IgG/anti-AQP4 antibody-positive patients show a significantly higher frequency of severe optic nerve damage (permanent complete blindness) than anti-AQP4 antibody-negative CMS patients [5,6]. Optic nerves are especially vulnerable to the detrimental effects of tissue edema in the optic canal where space is tight and increased tissue pressure easily causes circulatory insufficiency. In the spinal cord, the thoracic cord is prone to developing LESCLs in anti-AQP4 antibody-positive patients, although AQP4 is widely expressed from the cervical to sacral cord. Because the thoracic cord corresponds to the watershed of vascular supply in the spinal cord, even in the spinal cord, vulnerability to ischemia may be one of the factors contributing to the development of LESCLs. Prolongation of vasogenic edema at sites where the surrounding space is tight or the vascular supply is poor may cause poor recovery from tissue damage in patients with anti-AQP4 antibody.

## 15. Concluding remarks

The discovery of anti-AQP4 antibody has surely opened a new exciting research area in the field of human demyelinating diseases. There are two major hypotheses concerning the role of anti-AQP4 antibody: first, anti-AQP4 antibody directly causes NMO through astrocyte destruction, in a process that is distinct from that underlying MS; and second, anti-AQP4 antibody is a secondary modifying factor in OSMS and NMO. It remains to be elucidated whether MS and NMO are distinct diseases.

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# CSF chemokine alterations related to the clinical course of amyotrophic lateral sclerosis

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ABSTRACT

We measured the levels of 27 cytokines/chemokines and growth factors in cerebrospinal fluid (CSF) from 42 patients with sporadic amyotrophic lateral sclerosis (ALS), 12 patients with lower motor neuron disease (LMND), and 34 control patients with non-inflammatory neurological diseases (OND), using a multiplexed fluorescent bead-based immunoassay. Among cytokines/chemokines elevated in ALS, CCL2 and CXCL8 levels were negatively correlated with the revised ALS functional rating scale (ALSFRS-R) score, while CCL4 showed a positive correlation with ALSFRS-R score. CCL4 and CXCL10 showed negative correlations with disease progression rate. These chemokine alterations are assumed to somehow correlate with the clinical course of ALS.


## 1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease in which loss of motor neurons in the spinal cord, brainstem and motor cortex causes progressive paralysis. Studies using ALS model mice have reported that non-cell-autonomous cell death is a major contributor to motor neuron death (Boillée et al., 2006; Clement et al., 2003; Lobsiger and Cleveland, 2007; Yamanaka et al., 2008). Neuroglial inflammation is thus suggested to be crucial for motor neuron loss. Even in human sporadic ALS, increasing evidence suggests that certain cytokines/chemokines and growth factors, key mediators of both immune and neural networks, play critical roles in certain stages of ALS (Consilvio et al., 2004; McGeer and McGeer, 2002).

In human ALS, the levels of CCL2 (also known as macrophage chemoattractant protein-1) (Henkel et al., 2004; Wilms et al., 2003), interleukin (IL)-6 (Sekizawa et al., 1998), tumor necrosis factor (TNF)-α (Moreau et al., 2005; Poloni et al., 2000), and transforming growth factor (TGF)-β (Ilzecka et al., 2002) have been reported to be elevated in cerebrospinal fluid (CSF). We also measured the levels of 16 cytokines and chemokines in CSF from ALS patients by multiplexed fluorescent bead-based immunoassay, and found that CCL2, IL-5, and granulocyte-colony stimulating factor (G-CSF) are significantly elevated in patients with ALS compared with the levels in patients with non-inflammatory neurologic diseases (Tanaka et al., 2006). Among these, CCL2 showed a significant negative correlation with the revised ALS functional rating scale (ALSFRS-R) score, suggesting the possibility that this chemokine is a disease-aggravating factor (Tanaka et al., 2006). Recently, Mitchell et al (2009) reported that a variety of proinflammatory cytokines and growth factors, namely, CCL2, CCL3 (macrophage inflammatory protein-1α), CCL4 (macrophage inflammatory protein-1β), IL-2, IL-6, IL-15, and IL-17, G-CSF, vascular endothelial growth factor (VEGF), granulocyte-macrophage colony stimulating factor (GM-CSF), and basic fibroblast growth factor (bFGF), were all elevated in ALS patients' CSF. They reported that none of these had any significant correlation with clinical parameters, but that non-elevated CXCL8 (IL-8) had a weak negative correlation with the ALSFRS-R score. No biomarkers related to neuroprotection in ALS are known. Therefore, in the present study, we profiled CSF cytokines/chemokines and growth factors to identify those related to the clinical parameters of ALS and lower motor neuron disease (LMND).

## 2. Materials and methods

### 2.1. Patients

A total of 42 patients with sporadic ALS (20 males and 22 females; mean age ± standard deviation [SD] at examination, 56.7 ± 13.2 years) and 12 patients with sporadic LMND (six males and six females; 55.2 ± 15.7 years) were examined (Table 1). All patients with ALS were subjected to a thorough neurological examination and diagnosed as clinically definite or probable cases of ALS based on the El Escorial diagnostic criteria (Brooks, 1994) at the Department of Neurology,

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**Table 1**
Demographic features of patients with sporadic amyotrophic lateral sclerosis (ALS), lower motor neuron disease (LMND), and other non-inflammatory neurological diseases (OND).

| | ALS | LMND | OND |
|---|---|---|---|
| Number of patients | 42 | 12 | 34 |
| Sex (male/female) | 20/22 | 6/6 | 21/13 |
| Age at examination (mean ± SD, years) | 56.7 ± 13.2 | 55.2 ± 15.7 | 54.2 ± 12.9 |
| Disease duration (mean ± SD, months) | 13.0 ± 9.3 | 25.9 ± 28.6 | NA |
| Immunologic treatment (for the past years) | None | None | None |
| ALSFRS-R score (mean ± SD) | 39.0 ± 8.1 | 39.6 ± 6.72 | NA |
| *CSF* | | | |
| Cell count (mean ± SD, per μl) | 1.15 ± 1.04 | 1.27 ± 0.78 | 1.08 ± 1.11 |
| Total protein in CSF (mean ± SD, mg/dl) | 34.0 ± 14.3 | 42.4 ± 27.3 | 37.3 ± 17.7 |

Abbreviations in table: ALSFRS-R, revised amyotrophic lateral sclerosis functional rating scale; CSF, cerebrospinal fluid; NA, not applicable; SD, standard deviation.

Kyushu University Hospital, from 2000 to 2006. The mean disease duration at the time of CSF withdrawal was 13.0 ± 9.3 months in ALS patients and 25.9 ± 28.6 months in LMND patients. The disability level associated with the development and progression of ALS and LMND was determined using the revised ALS functional rating scale (ALSFRS-R) (Cedarbaum et al., 1999). The mean ALSFRS-R score was 39.0 ± 8.1 in ALS patients, and 39.6 ± 6.72 in LMND patients. The disease progression rate was defined as ALSFRS-R full score (48) − a patient's ALSFRS-R score/disease duration expressed in months. Thirty-four control patients with other non-inflammatory neurological diseases (OND) but no malignancies (21 males and 13 females; age at examination, 54.2 ± 12.9 years) examined during the same period were also enrolled. The OND group comprised 10 patients with cervical spondylosis, eight with sporadic spinocerebellar degeneration, four with lumbar herniation, four with metabolic neuropathy, two with hereditary spinocerebellar atrophy (SCA3 and unknown), and one each with spastic spinal paraplegia, drug-induced dystonia, peroneal nerve palsy, normal pressure hydrocephalus, Strüthers' ligament syndrome, senile blepharoptosis, and urge incontinence. No subjects were hypoxemic or undergoing any immunotherapies at the time of CSF drawing. The male-to-female ratio was not significantly different among these groups according to the chi-square test ($p > 0.1$). We compared the disease duration and the CSF total protein amounts between ALS and LMND patients using the Mann-Whitney U test. The disease duration was significantly longer in LMND patients than ALS patients ($p = 0.0149$), probably reflecting a slower disease course in the former, while the total CSF protein levels were not significantly different between the two groups ($p > 0.1$).

### 2.2. Cerebrospinal fluid collection

CSF samples were obtained by lumbar puncture from all patients and immediately centrifuged at 800 rpm at 4 °C for 5 min. The liquid phase of CSF that excluded the sedimented cells was stored at −80 °C until cytokine assay. CSF findings are shown in Table 1. No patients were considered to have systemic inflammation at the time CSF was drawn, because none had elevated serum C-reactive protein level or systemic autoantibodies, such as antinuclear antibody, SS-A and SS-B.

### 2.3. Multiplexed fluorescent bead-based immunoassay of CSF

The CSF liquid phase samples were simultaneously analyzed for 27 cytokines and chemokines, namely, IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, TNF-α, interferon (IFN)-γ, CCL2, CCL3, CCL4, CCL5 (regulated upon activation normal T-cell expressed and secreted), CCL11, CXCL8, CXCL10, G-CSF, GM-CSF, bFGF, platelet-derived growth factor-bb (PDGFbb), and VEGF, using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories, Hercules, CA), as described previously (Ishizu et al., 2005; Tanaka et al., 2006). Briefly, 50 μl of each CSF liquid and various concentrations of each cytokine standard (Bio-Rad) were added to 50 μl of antibody-conjugated beads (Bio-Rad) in 96-well filter plates (Millipore, Billerica, MA). Cytokine concentrations were calculated by reference to a standard curve for each cytokine derived using various concentrations of the cytokine standards (0.2, 0.78, 3.13, 12.5, 50, 200, 800 and 3200 pg/ml) assayed in the same manner as the CSF samples. The same batch of monoclonal antibodies for the Bio-Plex Cytokine Assay System was used throughout the experiments; the interassay and intraassay variabilities are reported to be less than 10% by the manufacturer (de Jager et al., 2003; Vignali, 2000). The detection limit for each cytokine was determined by recovery of the corresponding cytokine standard, and the lowest values with more than 70% recovery were set as the lower detection limits. The lower detection limits were as follows: 12.5 pg/ml for GM-CSF and IFN-γ, 3.13 pg/ml for IL-1ra, IL-2, IL-4, IL-6, IL-9, IL-13, IL-17, TNF-α, CCL2, CCL3, CCL11, CXCL10, G-CSF, bFGF, and VEGF, 0.78 pg/ml for IL-12(p70), CCL4, and PDGFbb, and 0.2 pg/ml for IL-1β, IL-5, IL-7, IL-10, IL-15, CCL5, and CXCL8. All samples were analyzed undiluted in duplicate.

### 2.4. Statistical analyses

We used the following statistical tests for appropriate applications. The non-parametric Kruskal–Wallis $H$ test was initially employed to compare the age at CSF withdrawal and CSF cytokine/chemokine levels among the studied group. When differences were significant, the Mann–Whitney $U$ test was used to determine the significance of differences between each group. For multiple comparisons, uncorrected $P$ values ($P^{uncorr}$) were corrected by multiplying them by the number of comparisons to calculate corrected $P$ values ($P^{corr}$) (Bonferroni–Dunn's correction). The disease duration and the CSF protein amounts were compared using the Mann–Whitney $U$ test. Spearman's rank correlation analysis was used to correlate various clinical parameters and CSF cytokine/chemokine levels which were significantly different among ALS, LMND and control. The male to female ratios were compared among the groups using the chi-square test. Statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Concentrations of each cytokine/chemokine in the liquid phase of CSF

Among the cytokines/chemokines measured, G-CSF, VEGF, CCL2, CCL4, CCL5, CCL11, CXCL8, CXCL10, TNF-α, IFN-γ, IL-1β, IL-7, IL-9, IL-12 (p70), and IL-17 levels were significantly higher in ALS than in OND patients (G-CSF: 9.670 ± 0.484 vs. 7.875 ± 0.537, $P^{corr} = 0.0005$; VEGF: 8.450 ± 0.676 vs. 4.855 ± 0.751, $P^{corr} = 0.0039$; CCL2: 276.755 ± 11.817 vs. 199.810 ± 13.134, $P^{corr} < 0.0001$; CCL4: 12.820 ± 0.974 vs. 7.700 ± 1.082, $P^{corr} = 0.0048$; CCL5: 0.845 ± 0.653 vs. 0.300 ± 0.726, $P^{corr} = 0.0165$; CCL11: 10.535 ± 0.551 vs. 8.395 ± 0.612 pg/ml, $P^{corr} = 0.0072$; CXCL8: 35.040 ± 1.498 vs. 24.335 ± 1.665, $P^{corr} < 0.0001$; CXCL10: 456.545 ± 42.442 vs. 289.760 ± 47.171, $P^{corr} < 0.0001$; TNF-α: 79.850 ± 3.266 vs. 61.125 ± 3.629, $P^{corr} = 0.0031$; IFN-γ: 24.370 ± 1.355 vs. 19.590 ± 1.506, $P^{corr} = 0.0132$; IL-1β: 0.955 ± 0.091 vs. 0.685 ± 0.101, $P^{corr} = 0.0348$; IL-7: 1.495 ± 0.075 vs. 1.125 ± 0.084, $P^{corr} = 0.0135$; IL-9: 27.090 ± 1.074 vs. 20.675 ± 1.193, $P^{corr} = 0.0020$; IL-12(p70): 6.900 ± 0.524 vs. 5.065 ± 0.582, $P^{corr} = 0.0339$; and IL-17: 2.700 ± 0.194 vs. 2.700 ± 0.215, $P^{corr} = 0.0027$) (Fig. 1). The levels of the other cytokines/chemokines did not differ significantly between the two groups. No significant difference was found between the OND and LMND groups in the levels of any of the cytokines/chemokines examined. We found no significant

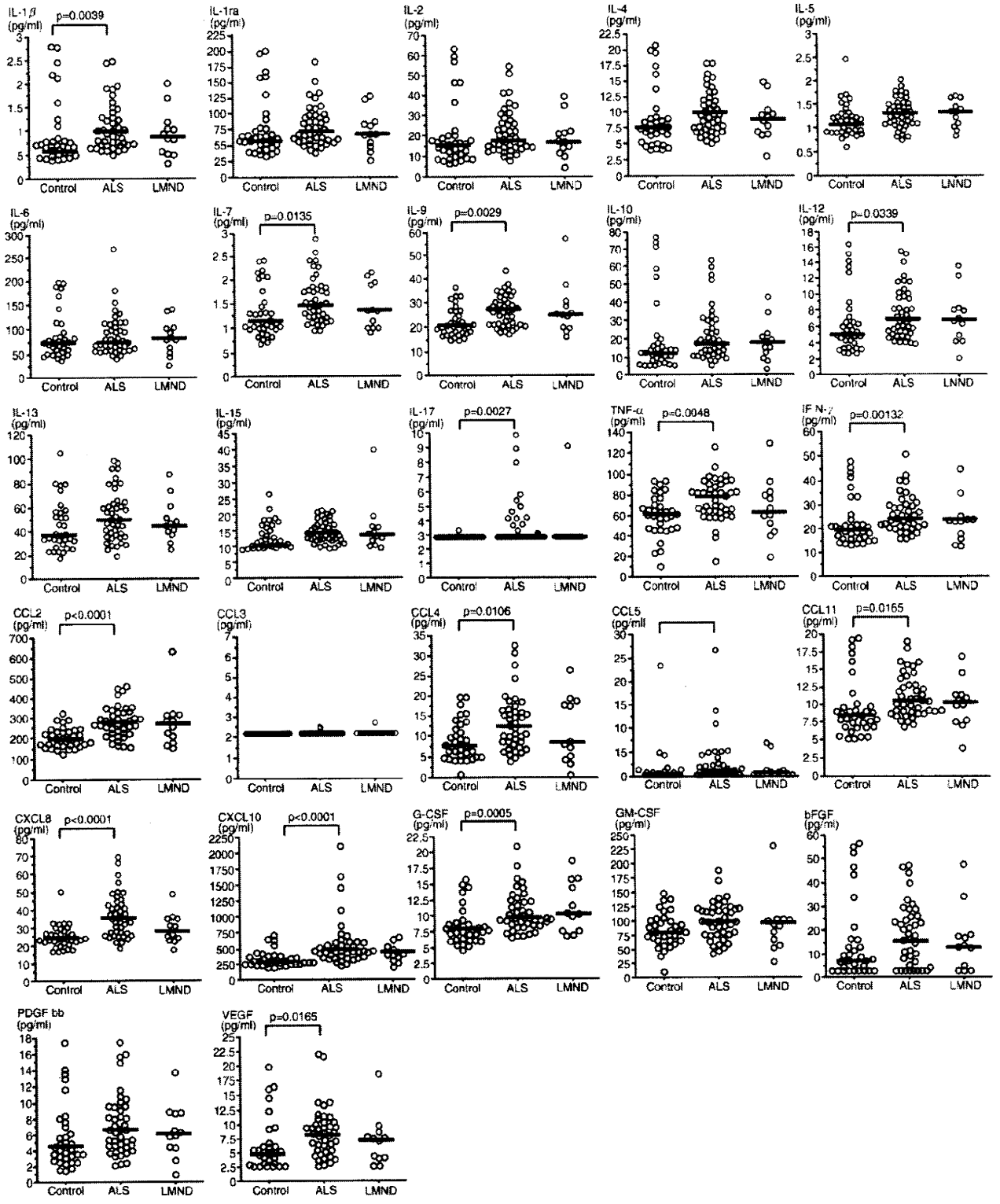


**Fig. 1.** Cytokine/chemokine levels in cerebrospinal fluid (CSF) supernatants from patients with amyotrophic lateral sclerosis (ALS) ($n=42$), lower motor neuron disease (LMND) ($n=12$), and other non-inflammatory neurological diseases (OND) ($n=34$) were measured using a multiplexed fluorescent bead-based immunoassay. Bars indicate the mean concentration in each group.

differences between the ALS and LMND patients, and the distributions and mean values of most cytokines/chemokines and growth factors, including VEGF in the LMND patients, showed similar trends to those in ALS patients; however, this similarity was not so obvious for some proinflammatory cytokines, namely TNF-α, CXCL8 and IL-17 (Fig. 1).

*3.2. Correlations between individual cytokine/chemokine levels and between each cytokine/chemokine level and various clinical parameters*

The concentration of CCL2 in CSF was negatively correlated with the ALSFRS-R score ($r=-0.390$, $P=0.0126$) and positively correlated with the CSF total protein level ($r=0.420$, $P=0.0071$) (Fig. 2). The concentration of CCL4 was positively correlated with the ALSFRS-R score ($r=0.354$, $P=0.0235$) and disease duration ($r=0.315$, $P=0.0435$), and negatively correlated with the disease progression rate ($r=-0.475$, $P=0.0024$). The concentration of

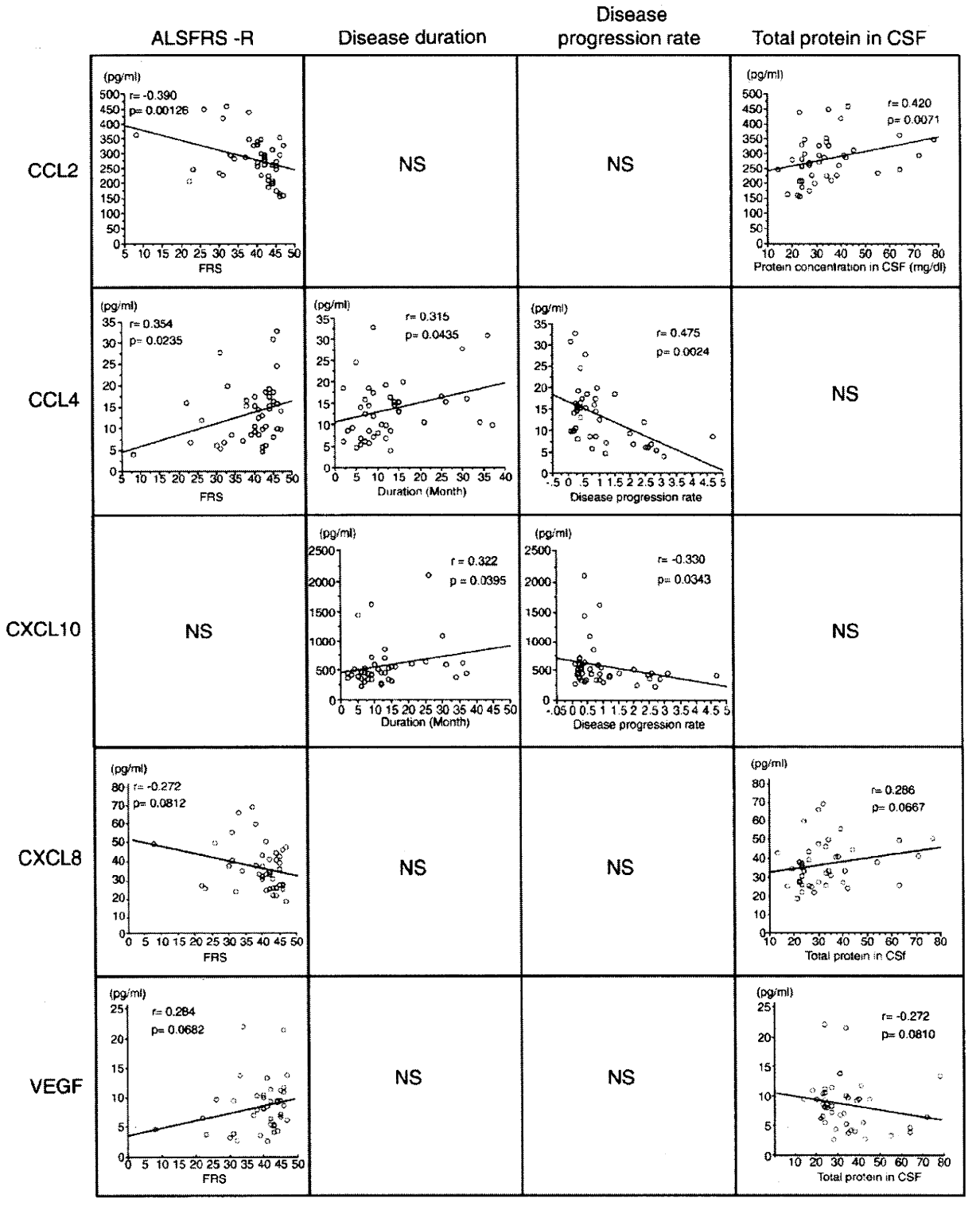


**Fig. 2.** Correlations between the concentrations of individual cytokines/chemokines in cerebrospinal fluid (CSF) and various clinical parameters. ALSFRS-R = revised amyotrophic lateral sclerosis functional rating scale. Abbreviations: CSF, cerebrospinal fluid; NS, not significant; VEGF, vascular endothelial growth factor.

CXCL10 was positively correlated with disease duration ($r=0.322$, $P=0.0395$) and negatively correlated with the disease progression rate ($r=-0.330$, $P=0.0343$). CXCL8 showed a tendencies for a negative correlation with the ALSFRS-R score ($r=-0.272$, $P=0.0812$) and for a positive correlation with the CSF total protein level ($r=0.286$, $P=0.0667$). VEGF tended to have a positive correlation with the ALSFRS-R score ($r=0.285$, $P=0.0682$) and a negative correlation with the CSF total protein level ($r=-0.272$, $P=0.0810$). CCL2 levels correlated positively with the levels of CXCL8 ($r=0.586$, $P=0.0002$), IL-9 ($r=0.490$, $P=0.0017$), IL-7 ($r=0.451$, $P=0.0039$), IFN-γ ($r=0.434$, $P=0.0055$), and CCL11 ($r=0.417$, $P=0.0076$), while CCL4 levels correlated positively with CXCL10 ($r=0.535$, $P=0.0006$) and VEGF ($r=0.440$, $P=0.0049$) levels (Table 2).

## 4. Discussion

In the present study we found that VEGF, G-CSF, IFN-γ, CCL2, CCL4, CCL5, CCL11, CXCL8, CXCL10, TNF-α, IL-1β, IL-7, IL-12, and IL-17 levels in CSF were elevated in ALS patients compared with OND patients. The main new findings concerning clinical correlation were that among cytokines/chemokines and growth factors elevated in ALS CSF, CCL2 and CXCL8 levels were negatively correlated with the ALSFRS-R score, whereas CCL4 and VEGF levels showed positive correlations with the ALSFRS-R score, and both CCL4 and CXCL10 levels showed negative correlations with disease progression rate.

Among the chemokines up-regulated in ALS CSF, CCL2 and CCL4 showed distinct patterns of correlation with clinical parameters: CCL2 had a positive correlation with disease severity whereas CCL4 had negative correlations with disease severity and progression rate. Furthermore, CCL2 and CCL4 showed distinct association patterns with proinflammatory cytokines in ALS CSF, suggesting that CCL2 and CCL4 interact with distinct cytokine/chemokine networks. Although both chemokines act on macrophages and microglia, their receptors are different: CCR2 is the main receptor for CCL2, while CCR5, a marker for type 1 helper T (Th1) cells, is also present on macrophages and is the main receptor for CCL4. Because the expression of both CCR2 and CCR5 is differentially regulated upon differentiation and activation in monocytes/macrophages (Kaufmann et al., 2001), these chemokines are thought to act on distinct subsets of monocyte lineage cells (Tacke and Randolph, 2006; Ubogu et al., 2006). Classical $CD14^{+}CD16^{-}$ monocytes express CCR2, while non-classical $CD14^{+/low}CD16^{+}$ ones express CCR5 and target Th1 immune responses (Tacke and Randolph, 2006; Weber et al., 2000).

We (Tanaka et al., 2006) and others (Baron et al., 2005; Henkel et al., 2004; Wilms et al., 2003) have reported increased levels of CCL2 in the CSF of ALS patients. Using distinct ALS patients in their early course, we confirmed our previous finding that CCL2 level is associated with disease severity (Tanaka et al., 2006). The significant positive correlation of CCL2 with disease progression rate found in our previous study was not obvious in the present one, probably because only ALS patients in the early course were enrolled this time. Observations that the CCL2 level was higher in CSF than in sera, that the levels in the two compartments were not correlated (Baron et al., 2005; Tanaka et al., 2006; Wilms et al., 2003), and that CCL2 production was enhanced in glial cells (Henkel et al., 2004), suggest that the main source of CCL2 is likely to be glial cells. CCL2 activates microglia, which then produce abundant proinflammatory cytokines/chemokines and inducible nitric oxide synthase, leading to the production of neurotoxic nitric oxide (Possel et al., 2000; Zhao et al., 2004). The positive correlation of CCL2 level with CSF protein amounts reflects a breakdown of the blood–brain barrier, supporting its role in glial inflammation. Therefore, it is reasonable to assume that CCL2 acts as a disease-aggravating factor in ALS. In addition, a tendency toward CXCL8 having a negative correlation with the ALSFRS-R score is consistent with the findings of Mitchell et al. (2009). Because CCL2 and CXCL8 levels were significantly positively correlated, these chemokines may constitute a neurotoxic cytokine network.

On the other hand, CCR5, a receptor for CCL4 and CCL5, is not only expressed on microglia, but also on neurons and astrocytes (Kaul and Lipton, 1999). CCL4 has been shown to be produced intrathecally by glial cells and to delay progression of HIV-associated dementia (Kaul and Lipton, 1999). The HIV envelope glycoprotein gp120 induces activation of macrophages and microglia (Kaul et al., 2001) and neuronal apoptosis in HIV-associated dementia (Brenneman et al., 1988); considered essential for its pronounced neurodegenerative effects (Kaul and Lipton, 1999). CCL4 together with CCL5 protects neurons from gp120-induced neuronal apoptosis (Kaul and Lipton, 1999) via an Akt-dependent signaling pathway in which neuronal Akt protects against excitotoxic insults (Kaul et al., 2007). Since excitotoxicity is also postulated to be operative in ALS (Heath and Shaw, 2002), intrathecally up-regulated CCL4 and CCL5 in ALS may represent a host defense mechanism. IFN-γ has recently been reported to enhance neurogenesis and neuroprotection in a mouse model of Alzheimer's disease (Baron et al., 2008), and to mediate neuroprotection against excitotoxic neural damage (Lee et al., 2006). Thus up-regulation of IFN-γ and the downstream molecule CXCL10 in ALS CSF may also represent a host's neuroprotective action in ALS. This may partly explain the significant negative correlation of CXCL10 with disease progression rate observed in the present study. A positive correlation of CCL4 and CXCL10 concentrations in CSF may thus be a reflection of a concerted host defense mechanism.

In the present study, a variety of proinflammatory cytokines/chemokines and growth factors were found to be up-regulated intrathecally in the early course of ALS. Patients with LMND also showed milder but similar trends for increases of proinflammatory cytokine/chemokines in CSF to those seen in ALS patients. It is possible that similar mechanisms may be partly operative in LMND, or alternatively, that some ALS patients might have been inappropriately included in the LMND group.

The delicate network of cytokines/chemokines and growth factors will already have been disturbed in the early course of motor neuron degeneration, with some up-regulated cytokines/chemokines such as CCL2 possibly neurotoxic, and others such as CCL4 possibly neuroprotective. Deciphering the complex actions of these altered cytokine/chemokine and growth factor networks may help the future elucidation of the pathogenesis of ALS.

**Table 2**
Summary of altered cytokines/chemokines in ALS CSF.

| Cytokines examined | Alteration | p value |
|---|---|---|
| IL-1β | Increase | $p=0.0348$ |
| IL-7 | Increase | $p=0.0135$ |
| IL-9 | Increase | $p=0.0020$ |
| IL-12(p70) | Increase | $p=0.0339$ |
| IL-17 | Increase | $p=0.0027$ |
| TNF-α | Increase | $p=0.0031$ |
| IFN-γ | Increase | $p=0.0132$ |
| CCL2 | Increase | $p<0.0001$ |
| CCL4 | Increase | $p=0.0048$ |
| CCL5 | Increase | $p=0.0165$ |
| CCL11 | Increase | $p=0.0072$ |
| CXCL8 | Increase | $p<0.0001$ |
| CXCL10 | Increase | $p<0.0001$ |
| G-CSF | Increase | $p=0.0005$ |
| VEGF | Increase | $p=0.0039$ |

The following cytokines/chemokines showed no significant alteration: IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-15, CCL3, GM-CSF, and PDGFbb.

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# Reappraisal of brain MRI features in patients with multiple sclerosis and neuromyelitis optica according to anti-aquaporin-4 antibody status

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ABSTRACT

Brain lesions are not uncommon in neuromyelitis optica (NMO) patients with anti-aquaporin-4 (AQP4) antibody; however, the appearance of these lesions is said to be different from that of those in Western patients with multiple sclerosis (MS). To clarify the similarities and dissimilarities of brain lesions in anti-AQP4 antibody-positive and -negative MS and NMO patients, we examined the presence of anti-AQP4 antibody in the sera of 148 consecutive patients fulfilling Poser's criteria for clinically definite MS, of whom 38 also met the revised NMO criteria, using an immunofluorescence method, and analyzed brain lesions by magnetic resonance imaging (MRI). Brain lesions fulfilling the Barkhof criteria were significantly more common in 121 patients without anti-AQP4 antibody than in 27 patients with anti-AQP4 antibody (57.0% vs. 33.3%, $P=0.033$), while the frequency of those that met the Paty criteria was not different between the two groups (74.4% vs. 73.5%). Ovoid lesions were detected more commonly in patients without anti-AQP4 antibody than in those with the antibody (72.3% vs. 48.2%, $P=0.022$). The anti-AQP4 antibody-positive patients had significantly more atypical brain lesions, such as extensive brain lesions, than the anti-AQP4 antibody-negative ones (18.5% vs. 1.7%, $P=0.0023$). Thus, although MS-like brain lesions are more common in anti-AQP4 antibody-negative patients than anti-AQP4 antibody-positive patients, approximately 30 to 50% of patients with anti-AQP4 antibody harbour brain MRI lesions indistinguishable from those present in typical MS patients, such as periventricular ovoid lesions, suggesting the existence of considerable overlap in brain MRI features between anti-AQP4 antibody-positive and -negative Asian patients. In the present study, NMO patients with brain lesions showed a significantly higher annualized relapse rate ($P^{corr}=0.017$) and higher frequency of anti-AQP4 antibody ($P^{corr}<0.0001$) than typical NMO patients without brain lesions, suggesting that development of brain lesions in NMO may reflect high disease activity and thus be a warning sign.



## 1. Introduction

Neuromyelitis optica (NMO) is characterised by severe and selective involvement of the optic nerves and spinal cord, which frequently shows longitudinally extensive spinal cord lesions (LESCLs) extending over three or more vertebral segments. NMO was originally defined as a disease involving only the optic nerves and spinal cord with a monophasic course [1]. However, the concept of NMO has changed along with the collection and analysis of samples from many NMO patients, and a relapsing form of NMO is widely accepted in Western countries [2,3]. Additionally, a highly specific IgG against NMO, designated NMO-IgG, has been described [4], and its relevant antigen was reported to be aquaporin-4 (AQP4) [5]. The presence of NMO-IgG/anti-AQP4 antibody has also influenced the concept of NMO. Some patients with NMO-IgG also show atypical brain lesions, such as large confluent lesions (>3 cm) and diencephalic lesions, during their clinical course [6]. In fact, 60% of patients fulfilling the 1999 criteria for NMO show brain lesions on MRI [6]. Thus, based on such evidence, the 2006 revised criteria for NMO include the presence of NMO-IgG and do not preclude patients with brain lesions [3].

The distribution of atypical brain lesions on MRI reflects the distribution of high expression of AQP4, and histopathological analyses of NMO show perivascular IgM and IgG deposition with complement activation and loss of immunoreactivity to AQP4 [7–9]. These findings suggest a role for humoral immunity in the pathogenesis of NMO and a direct etiological role for NMO-IgG.

However, either NMO-IgG or anti-AQP4 antibody is detected in around 10% of MS patients who fulfil the established clinical criteria

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for MS [4,10,11]. We have also reported that anti-AQP4 antibody-positive patients occasionally have MS-like brain lesions, such as periventricular ovoid lesions [11]. Thus, it remains to be elucidated whether brain MRI lesions are distinguishable between MS and NMO patients, and between those with and without anti-AQP4 antibody, or if considerable overlaps exist between these conditions. Because the opticospinal form of MS (OSMS) in Asians is now claimed to be the same as NMO in Westerners, it is especially problematic to differentiate NMO from MS in Asian patients, who frequently show severe and selective involvement of the optic nerve and spinal cord, irrespective of the presence or absence of anti-AQP4 antibody [11]. Therefore, it is critical to compare brain MRI lesions in a large unbiased series of Asian MS and NMO patients. In the present study, we aimed to compare the frequencies of typical MS-like brain lesions and atypical ones between Japanese MS and NMO patients, and between those with and without anti-AQP4 antibody.

## 2. Methods

### 2.1. Patients

For the present study, we enrolled 148 consecutive patients with clinically definite relapsing–remitting or secondary-progressive MS based on the Poser criteria [12], seen at the MS clinic of the Department of Neurology, Kyushu University Hospital during 1987–2007, and whose sera were available for anti-APQ4 antibody assays. From a retrospective review of the medical records of all patients, we recorded demographic and imaging data. There were 137 relapsing–remitting and 11 secondary-progressive MS patients. All patients were residents of Kyushu Island, the southernmost part of mainland Japan. None were seropositive for human T cell leukaemia virus type I. No patients with primary progressive MS were included in the present study. Patients with monophasic NMO without subsequent relapse were also excluded to avoid including patients with acute disseminated encephalomyelitis. Among the 148 patients, 27 (18.2%, 24 female and 3 male) were positive for anti-AQP4 antibody, and 38 also met the revised Wingerchuk's criteria for NMO [3]. We then classified these NMO patients into "typical NMO" and "NMO with brain lesions" based on whether they had brain lesions meeting the Paty criteria [13]. We adopted the Paty criteria [13] because in the Wingerchuk's revised criteria for NMO [3] the Paty criteria were recommended for determining the presence of MS-like brain lesions. Therefore, "typical NMO" patients were those who showed only optic neuritis and myelitis without brain lesions fulfilling the Paty criteria [13]. "NMO patients with brain lesions" were those who had only optic neuritis and myelitis and also had brain lesions fulfilling the Paty criteria [13]. "MS patients" were those who met the Poser [12] and the revised McDonald criteria [14] and did not meet the definition of either "typical NMO" or "NMO with brain lesions".

### 2.2. Magnetic resonance imaging

All MRI studies were performed using 1.5-T Magnetom Vision and Symphony units (Siemens Medical Systems, Erlangen, Germany) as described previously [15]. The typical imaging parameters for the brain were as follows: axial T2-weighted turbo spin-echo imaging using TR/TE = 2800/90 ms, flip angle = 180°; axial turbo-fluid-attenuated inversion recovery (FLAIR) imaging using TI/TR/TE = 2200/9000/110 ms, flip angle = 180; and sagittal and axial pre-contrast and axial and coronal post-contrast T1-weighted spin-echo imaging using TR/TE range = 400–460/12–17 ms, and flip angle range = 80–90°. One excitation, with a matrix of 256 × 256, slice thickness of 5 mm, and slice gap of 2.5 mm, was used for all brain studies. Gadopentetate dimeglumine at 0.1 mmol/kg body weight was administered intravenously for contrast-enhanced studies.

MRI scans were taken at the time of clinical relapse (within 30 days of the onset of acute exacerbation) or in the remission phase. Brain MRI scans from 87 patients at relapse and 136 patients in remission were examined. Brain MRI lesions were evaluated according to the Barkhof criteria [16] and Paty criteria [13] for MS. Atypical brain lesions, such as extensive brain lesions (>3 cm), bilateral diencephalic (thalamic/hypothalamic) lesions, cavity formation and extension from the cervical cord into the brainstem were defined based on previous reports [6,11]. At the time of brain MRI, treatment was being received by the patients being imaged in 108 out of 223 scans (58 on IFNβ-1b, 42 within one month of steroid pulse therapy and eight on both).

### 2.3. Anti-AQP4 antibody assay

Green fluorescence protein (GFP)-AQP4 fusion protein–transfected human embryonic kidney cells (HEK-293) were produced as previously described [11]. AQP4-expressing cells were initially incubated with human serum samples diluted 1:4 with DMEM for 1 h at 37.0 °C without cell fixation, washed in phosphate-buffered saline, and then visualized with an Alexa 594-conjugated goat anti-human IgG antibody (Invitrogen). The fluorescence of non-fixed cells was observed using a confocal laser-scanning microscope (FLUOVIEW FV300; Olympus Optical Co., Tokyo, Japan). The anti-AQP4 antibody assay was carried out at least twice for each sample, and those that gave a positive result twice were deemed to be positive.

### 2.4. Statistical analysis

Statistical analyses of numerical variables were performed using the Mann–Whitney $U$ test. Differences in frequencies between the two subgroups were tested for significance using Fisher's exact probability test. When multiple comparisons were performed, uncorrected $P$ values ($P^{uncorr}$) were corrected by multiplying them by the number of comparisons (Bonferroni–Dunn's correction) to calculate corrected $P$ values ($P^{corr}$).

**Table 1**
Comparison of demographic features between patients with NMO and those with MS.

| | NMO patients ($n=38$) | MS patients ($n=110$) |
|---|---|---|
| No. of females/males | 32/6 (5.3:1) | 75/35 (2.1:1) |
| Age at onset (years)[a] | 35.4 ± 14.2 | 31.3 ± 12.4 |
| Disease duration (years)[a] | 11.8 ± 8.7 | 11.9 ± 9.9 |
| Annualized relapse rate[a] | 0.99 ± 0.58* | 0.68 ± 0.58* |
| EDSS score[a] | 5.2 ± 2.6* | 3.4 ± 2.6* |
| Anti-AQP4 antibody | 24/38 (63.2%)* | 3/110 (2.7%)* |
| Frequency of symptoms: | | |
| Optic neuritis | 38/38 (100.0%)* | 62/110 (56.7%)* |
| Bilateral optic neuritis | 6/38 (15.8%) | 13/110 (11.9%) |
| Severe optic neuritis (≥FS 5) | 28/38 (73.7%)* | 38/110 (34.5%)* |
| Myelitis | 38/38 (100.0%) | 91/110 (82.7%) |
| Acute transverse myelitis | 23/38 (60.5%)* | 22/110 (20.0%) |
| Secondary progression | 0/38 (0.0%) | 11/110 (10.0%) |
| CSF: | | |
| Marked pleocytosis (≥50/μl) | 4/35 (11.4%) | 6/101 (5.9%) |
| Neutrophilia (≥5/μl) | 4/35 (11.4%) | 4/94 (4.3%) |
| OB | 5/34 (14.7%)* | 37/90 (41.1%)* |
| IgG index (≥0.658)[b] | 14/33 (42.4%) | 42/83 (50.6%) |
| LESCLs during the entire course | 32/38 (84.2%)* | 28/110 (25.5%)* |

AQP4 = aquaporin-4; CNS = central nervous system; CSF = cerebrospinal fluid; EDSS = Kurtzke's Expanded Disability Status Scale [17]; FS = Kurtzke's Visual Functional Scale [17]; LESCLs = longitudinally extensive spinal cord lesions; MS = multiple sclerosis; NMO = neuromyelitis optica; OB = oligoclonal IgG bands.

[a] Means ± SD.

[b] The upper normal range of IgG index was derived from our previous study [24].

* $P<0.05$.

**Table 2**
Comparison of demographic features among patients with typical NMO, patients with NMO with brain lesions, and patients with MS.

| | Typical NMO patients ($n=21$) | NMO with brain lesions ($n=17$) | MS patients ($n=110$) |
|---|---|---|---|
| No. of females/males | 18/3 (5.7:1) | 14/3 (5:1) | 75/35 (2.1:1) |
| Age at onset (years)[a] | 33.4 ± 14.5 | 37.8 ± 14.0 | 31.3 ± 12.4 |
| Disease duration (years)[a] | 11.6 ± 8.8 | 12.1 ± 8.7 | 11.9 ± 9.9 |
| Annualized relapse rate[a] | 0.77 ± 0.43* | 1.26 ± 0.62*,** | 0.68 ± 0.58** |
| EDSS score[a] | 5.6 ± 2.3* | 4.7 ± 2.9 | 3.4 ± 2.6* |
| Anti-AQP4 antibody | 7/21 (33.3%)*,*** | 17/17 (100.0%)*,** | 3/110 (2.7%)**,*** |
| Frequency of symptoms: | | | |
| Optic neuritis | 21/21 (100.0%)* | 17/17 (100.0%)** | 62/110 (56.6%)*,** |
| Bilateral optic neuritis | 4/21 (19.0%) | 2/17 (11.8%) | 13/110 (11.9%) |
| Severe optic neuritis (≥FS 5) | 14/21 (66.7%)* | 14/17 (82.3%)** | 38/110 (34.5%)*,** |
| Myelitis | 21/21 (100.0%) | 17/17 (100.0%) | 91/110 (82.7%) |
| Acute transverse myelitis | 15/21 (71.4%)* | 8/17 (47.1%) | 22/110 (20.0%)* |
| Secondary progression | 0/21 (0.0%) | 0/17 (0.0%) | 11/110 (10.0%) |
| CSF: | | | |
| Marked pleocytosis (≥50/μl) | 1/18 (5.6%) | 3/17 (17.6%) | 6/101 (5.9%) |
| Neutrophilia (≥5/μl) | 1/18 (5.6%) | 3/17 (17.6%) | 4/94 (4.3%) |
| OB | 3/18 (16.7%) | 2/16 (12.5%) | 37/90 (41.1%) |
| IgG index (≥0.658)[b] | 8/17 (47.1%) | 6/16 (37.5%) | 42/83 (50.6%) |
| LESCLs during the entire course | 17/21 (81.0%)* | 15/17 (88.2%)** | 28/110 (25.5%)*,** |

AQP4 = aquaporin-4; CSF = cerebrospinal fluid; EDSS = Kurtzke's Expanded Disability Status Scale [17]; FS = Kurtzke's Visual Functional Scale [17]; LESCLs = longitudinally extensive spinal cord lesions; MS = multiple sclerosis; NMO = neuromyelitis optica; OB = oligoclonal IgG bands.
*, **, *** Corrected $P<0.05$.
[a] Means ± SD.
[b] The upper normal range of IgG index was derived from our previous study [24].

## 3. Results

### 3.1. Demographic features

The demographic features of the 148 patients are summarized in Table 1. The disease duration was similar between NMO and MS patients. Although relapse rate, Kurtzke's Expanded Disability Status Scale (EDSS) scores [17], and frequencies of severe optic neuritis, ATM, and LESCLs during the entire course were significantly greater in the 38 patients who satisfied the revised NMO criteria [3] than in the remaining 110 MS patients, the frequency of oligoclonal bands (OB) was significantly higher in MS patients than that in NMO patients. Among MS patients, 65.2% had OBs and/or an elevated IgG index (OB/high IgG index). Although none of the four MS patients with CSF neutrophilia had anti-AQP4 antibodies, all of them had LESCLs.

When clinical features were compared among patients with typical NMO, NMO with brain lesions and MS, EDSS score and frequencies of severe optic neuritis, ATM and LESCLs during the entire course were significantly greater in typical NMO patients than in MS patients ($P^{corr}=0.0024$, $P^{corr}=0.023$, $P^{corr}<0.0001$, and $P^{corr}<0.0001$, respectively) (Table 2). Annualized relapse rates were significantly higher in NMO patients with brain lesions than in MS patients ($P^{corr}<0.001$) and typical NMO patients ($P^{corr}=0.017$). Frequencies of severe optic neuritis and LESCLs were also significantly higher in NMO patients with brain lesions than in MS patients ($P^{corr}<0.001$ and $P^{corr}<0.001$, respectively). Anti-AQP4 antibody positivity rate was highest in NMO patients with brain lesions and the rate was significantly higher in NMO patients with brain lesions (100%) than typical NMO patients (33.3%, $P^{corr}<0.0001$) and MS patients (2.7%, $P^{corr}<0.0001$). It was also significantly higher in

**Table 3**
Comparison of demographic features between anti-AQP4 antibody-positive and -negative patients with NMO and MS.

| | Anti-AQP4 antibody-positive patients ($n=27$) | Anti-AQP4 antibody negative patients ($n=121$) |
|---|---|---|
| No. of female/male patients | 24/3 (8.0:1)* | 83/38 (2.2:1)* |
| Age at onset (years)[a] | 36.3 ± 13.8 | 31.4 ± 12.7 |
| Disease duration (years)[a] | 13.7 ± 9.2 | 11.5 ± 9.6 |
| Annualized relapse rate[a] | 1.0 ± 0.62* | 0.71 ± 0.57* |
| EDSS score[a] | 4.7 ± 2.6 | 3.7 ± 2.7 |
| Frequency of symptoms: | | |
| Optic neuritis | 27/27 (100.0%)* | 72/121 (59.5%)* |
| Bilateral optic neuritis | 4/27 (14.8%) | 15/121 (12.4%) |
| Severe optic neuritis (FS ≥ 5) | 21/27 (77.8%)* | 45/121 (37.2%)* |
| Myelitis | 26/27 (96.3%) | 103/121 (85.1%) |
| Acute transverse myelitis | 12/27 (44.4%) | 33/121 (27.3%) |
| CSF: | | |
| Marked pleocytosis (≥50/μl) | 3/25 (12.0%) | 7/111 (6.3%) |
| Neutrophilia (≥5/μl) | 3/25 (12.0%) | 5/104 (4.8%) |
| OB | 5/24 (20.8%) | 37/100 (37.0%) |
| IgG index (≥0.658)[b] | 9/23 (39.1%) | 47/93 (50.5%) |
| LESCLs during the entire course | 20/27 (74.1%)* | 40/121 (33.1%)* |

AQP4 = aquaporin-4; CSF = cerebrospinal fluid; EDSS = Kurtzke's Expanded Disability Status Scale [17]; FS = Kurtzke's Visual Functional Scale [17]; LESCLs = longitudinally extensive spinal cord lesions; MS = multiple sclerosis; NMO = neuromyelitis optica; OB = oligoclonal IgG bands.
[a] Means ± SD.
[b] The upper normal range of IgG index was derived from our previous study [24].
* $P<0.05$.

**Table 4**
Comparison of brain MRI findings between NMO and MS.

| | NMO patients ($n=38$) | MS patients ($n=110$) |
|---|---|---|
| Barkhof brain lesions[a] | 7/38 (18.4%)* | 71/110 (64.6%)* |
| ≥9 T2 brain lesions | 8/38 (21.1%)* | 76/110 (69.1%)* |
| ≥1 Gd-enhanced lesion | 3/36 (8.3%)* | 35/108 (32.4%)* |
| ≥1 juxtacortical lesion | 15/38 (39.5%)* | 81/110 (73.6%)* |
| ≥1 periventricular lesion | 11/38 (29.0%)* | 81/110 (73.6%)* |
| ≥1 infratentorial lesion | 13/38 (34.2%)* | 72/110 (65.5%)* |
| Paty brain lesions[b] | 17/38 (44.7%)* | 92/110 (83.6%)* |
| Ovoid lesions | 12/38 (31.6%)* | 87/108 (80.6%)* |
| Atypical brain lesions | 10/38 (26.3%) | 23/110 (20.9%) |
| Extensive brain lesions | 5/38 (13.2%)* | 2/110 (1.8%)* |
| Bil. diencephalic lesions | 0/38 (0.0%) | 6/110 (5.5%) |
| Cavity formation | 3/38 (7.9%) | 16/110 (14.6%) |
| Extension from the cervical cord into brainstem | 3/38 (7.9%)* | 0/110 (0.0%)* |

Bil. = bilateral; Gd = gadolinium; MS = multiple sclerosis; NMO = neuromyelitis optica.
[a] Brain lesions fulfilling the Barkhof criteria [16].
[b] Brain lesions fulfilling the Paty criteria [13].
* $P<0.05$.

typical NMO patients than MS patients ($P^{corr}<0.001$). The frequencies of OBs and an elevated IgG index were higher in MS patients than in typical NMO patients and NMO patients with brain lesions, but the differences did not reach statistical significance (Table 2).

The demographic features of patients with and without anti-AQP4 antibody are shown in Table 3. Female to male ratio, annualized relapse rate and frequencies of optic neuritis, severe optic neuritis, and LESCLs during the entire course, were significantly higher in anti-AQP4 antibody-positive patients than in anti-AQP4 antibody-negative patients ($P=0.034$, $P=0.0070$, $P<0.0001$, $P=0.00018$, and $P=0.00015$, respectively).

### 3.2. Brain MRI findings

The frequencies of brain lesions fulfilling the Barkhof [16] or Paty [13] criteria were significantly higher in MS patients than in NMO patients (64.6% vs. 18.4%, $P<0.0001$, and 83.6% vs. 44.7%, $P<0.0001$, respectively). The frequency of ovoid lesions was similarly higher in MS patients than that in NMO patients (80.6% vs. 31.6%, $P<0.001$). Although the frequency of total atypical brain lesions was not different between the two groups, extensive brain lesions (13.2% vs. 1.8%, $P=0.012$) and lesions extending from the cervical cord into the brainstem (7.9% vs. 0.0%, $P=0.016$) were significantly more common in NMO patients than in MS patients (Table 4).

When brain MRI features were compared among typical NMO patients, NMO patients with brain lesions, and MS patients, atypical brain lesions were most frequently found in NMO patients with brain lesions (Table 5). The frequencies of atypical brain lesions and extensive brain lesions were significantly higher in the NMO patients with brain lesions (52.9% and 29.4%, respectively) than in MS patients (20.9%, $P^{corr}=0.038$, and 1.8%, $P^{corr}=0.0013$, respectively) and typical NMO patients (4.8%, $P^{corr}=0.0056$, and 0%, $P^{corr}=0.037$ respectively). By contrast, ovoid lesions were significantly more commonly found in MS patients (80.6%) and NMO patients with brain lesions (64.7%) than in typical NMO patients (4.8%, $P^{corr}<0.001$ and $P^{corr}<0.001$, respectively).

In comparisons between those with and without anti-AQP4 antibody, brain lesions fulfilling the Barkhof criteria were significantly less common in anti-AQP4 antibody-positive patients (33.3%) than in anti-AQP4 antibody-negative patients (57.0%) ($P=0.033$), while fulfilment of the Paty criteria during the entire clinical course was observed nearly as frequently in anti-AQP4 antibody-positive patients as in anti-AQP4 antibody-negative ones (74.1% vs. 73.5%) (Table 6). Among the items of Barkhof's criteria, the frequency of patients having ≥nine T2 hyperintense lesions was significantly higher among anti-AQP4 antibody-negative patients than among anti-AQP4 antibody-positive patients ($P=0.031$) while the frequencies of patients with ≥1 gadolinium-enhanced lesion, those with ≥1 juxtacortical lesion, those with ≥1 periventricular lesion and those with ≥1

**Table 5**
Comparison of brain MRI findings among patients with typical NMO, NMO with brain lesions and MS.

| | Typical NMO patients ($n=21$) | NMO with brain lesions ($n=17$) | MS patients ($n=110$) |
|---|---|---|---|
| Barkhof brain lesions[a] | 0/21 (0.0%)*,** | 7/17 (41.2%)** | 71/110 (64.6%)* |
| ≥9 T2 brain lesions | 0/21 (0.0%)*,** | 8/17 (47.1%)** | 76/110 (69.1%)* |
| ≥1 Gd-enhanced lesion | 1/19 (5.3%)* | 2/17 (11.8%) | 35/108 (32.4%)* |
| ≥1 juxtacortical lesion | 6/21 (28.6%)* | 9/17 (52.9%) | 81/110 (73.6%)* |
| ≥1 periventricular lesion | 0/21 (0.0%)*,** | 11/17 (64.7%)** | 81/110 (73.6%)* |
| ≥1 infratentorial lesion | 3/21 (14.3%)*,** | 10/17 (58.8%)** | 72/110 (65.5%)* |
| Paty brain lesions[b] | 0/21 (0.0%)*,** | 17/17 (100.0%)** | 92/110 (83.6%)* |
| Ovoid lesions | 1/21 (4.8%)*,** | 11/17 (64.7%)** | 87/108 (80.6%)* |
| Atypical brain lesions | 1/21 (4.8%)* | 9/17 (52.9%)*,** | 23/110 (20.9%)** |
| Extensive brain lesions | 0/21 (0.0%)* | 5/17 (29.4%)*,** | 2/110 (1.8%)** |
| Cavity formation | 0/21 (0.0%) | 3/17 (17.7%) | 16/110 (14.6%) |
| Bil. diencephalic lesions | 0/21 (0.0%) | 0/17 (0.0%) | 6/110 (5.5%) |
| Extension from the cervical cord into brainstem | 1/21 (4.8%) | 2/17 (11.8%) | 0/110 (0.0%) |

Bil. = bilateral; Gd = gadolinium; MS = multiple sclerosis; NMO = neuromyelitis optica.
*, ** Corrected $P<0.05$.
[a] Brain lesions fulfilling the Barkhof criteria [16].
[b] Brain lesions fulfilling the Paty criteria [13].