A Clinical/Immunological Neuromyelitis Optica Association Study

by

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Abstract

Neuromyelitis optica (NMO) and NMO spectrum disorders (NMOSD) are associated with the disease-specific autoantibody aquaporin-4 (AQP4-Ab), which is thought to be pathogenic. Some NMO/NMOSD patients do not have this antibody and may have different clinical and immuno-pathological disease characteristics, but previous clinical NMO/NMOSD studies have been confounded by inclusion of such patients.

To define better the characteristics of AQP4-Ab disease, disease course, outcomes and predictors of disability were investigated in 106 AQP4-Ab positive NMO/NMOSD patients from the UK and Japan. AQP4-Ab positivity conferred a high risk of relapsing disease and substantial disability; age at disease onset and ethnicity were important predictors of disability type. Visual disability was more common in younger patients and those of Afro-Caribbean ethnicity whilst older patients and Caucasian patients were more at risk of motor disability.

To determine whether disease characteristics were influenced by AQP4-Ab binding specificities, the differential binding of patient AQP4-Ab against the two main AQP4 isoforms was investigated. Although the relative binding to the two isoforms differed between patients, there was no association between these differences and clinical features such as relapse type, severity, onset age and ethnicity.

The clinical and *in vitro* characteristics of AQP4-Ab negative NMO/NMOSD patients were studied. It was shown that these patients represent an aetiologically heterogeneous group. Some have other inflammatory and infectious disorders, some have low levels of AQP4-Ab and a significant proportion have antibodies to myelin oligodendrocyte glycoprotein (MOG-Ab). Others have antibodies that bind to neurons or oligodendrocytes in primary cultures. Attempts to identify novel antigens by immunocapture techniques were made, but were not successful. Patients with MOG-Ab showed differences when compared to those with AQP4-Ab including higher proportion of males, younger age at disease onset and greater likelihood of conus involvement on imaging. Additionally, patients with MOG-Ab appear to have more favourable outcomes, with better improvement from the onset attack and a lower probability of subsequent relapses.

In conclusion, the work in this thesis has shown that AQP4-Ab disease is not synonymous with the term NMO and that seronegative NMO/NMOSD patients represent a clinically and aetiologically heterogeneous group and should therefore be classed separately from those with AQP4-Ab.

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List of Abbreviations

Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADEM	Acute disseminated encephalomyelitis
AMPAR	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptor
ANA	Anti-nuclear antibodies
AQP4	Aquaporin-4
AQP4-Ab	Aquaporin-4 antibodies
ARR	Annualised relapse rate
BAFF	B-cell activating factor
BSA	Bovine serum albumin
CASPR-2	Contactin associated protein-2
CBA	Cell-based assay
CDC	Complement-dependent cytotoxicity
CI	Confidence interval
CIS	Clinically isolated syndrome
CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CRMP5	Collapsin response mediator protein 5
CSF	Cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified Eagle’s growth medium
DNA	Deoxyribonucleic acid
EAAT2	Excitatory amino acid transporter-2
EAE	Experimental autoimmune encephalomyelitis
ECL	Enhanced chemiluminescence
EDMUS	European Database System for Multiple Sclerosis
EDSS	Expanded Disability Status Scale
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENA	Extractable nuclear antigens
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate

FU	Follow up
$GABA_BR$	Gamma-aminobutyric acid B receptor
GFAP	Glial fibrillary acidic protein
GlyR	Glycine receptor
GlyR-Ab	Glycine receptor antibodies
HBSS	Hanks' Balanced Salt Solution
HEK293	Human embryonic kidney 293
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulphonic acid)
HR	Hazard ratio
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IIF	Indirect immunofluorescence
LB	Luria-Bertani
LDS	Lithium dodecyl sulphate
LETM	Longitudinally extensive transverse myelitis
LGI1	Leucine-rich glioma inactivated 1
M1R	HEK293 cells transfected with M1 AQP4 and dsred
M23R	HEK293 cells transfected with M23 AQP4 and dsred
M23U	HEK293 cells transfected with M23 AQP4 and no dsred
MAP-2	Microtubule-associated protein-2
MBP	Myelin basic protein
MEM	Minimum essential medium
MES	2-(N-morpholino) ethanesulfonic acid
MFI	Median fluorescence intensity
MG	Myasthenia gravis
MOG	Myelin oligodendrocyte glycoprotein
MOG-Ab	Myelin oligodendrocyte glycoprotein antibodies
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NB	Neurobasal
NB+++	Neurobasal supplemented with B27, glutamine and PSA
NK	Natural killer
NMDA	N-methyl-D-asparatate
NMDAR	N-methyl-D-asparatate receptor

NMO	Neuromyelitis optica
NMO-IgG	Human sera containing NMO antibodies
NMOSD	Neuromyelitis optica spectrum disorder
OAP	Orthogonal arrays of particles
OCB	Oligoclonal bands
ON	Optic neuritis
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBS(T)	Phosphate-buffered saline containing 0.0025% Triton X-100
PE	R-phycoerythrin
PEI	Polyethylenemine
PLL	Poly-L-Lysine
PMSF	Phenylmethylsulfonyl fluoride
PSA	Penicillin/streptomycin/ampicillin
rAb	Recombinant monoclonal antibody
s.d.	Standard deviation
SDS	Sodium dodecyl sulphate
SEPs	Sensory evoked potentials
SLE	Systemic lupus erythematosus
TM	Transverse myelitis
VEPs	Visual evoked potentials

Chapter 1: Introduction

1.1 Introduction

In 1894, the French physician Eugene Devic described a case of subacute myelitis followed quickly by optic neuritis (ON), and his student Fernand Gault wrote a thesis describing 16 other published cases of co-existing ON and transverse myelitis (TM) (Devic, 1894). Thereafter, the term Devic's disease was adopted to describe patients with monophasic inflammation of the optic nerves and spinal cord occurring simultaneously or sequentially. Following descriptions of patients with relapsing-remitting disease and temporal separation of ON and TM (Beck, 1927; McAlpine,1938) use of the term expanded to encompass such patients. Over the years, the term 'Devic's disease' has been largely superseded by the more descriptive name of neuromyelitis optica (NMO). NMO describes inflammatory demyelination of the central nervous system (CNS), primarily causing attacks of ON and TM, which are often recurrent. The myelitis is typically associated with extensive signal change on MRI; this is widely termed longitudinally extensive transverse myelitis (LETM).

Until recently, NMO was considered a severe form of multiple sclerosis (MS) in Asia and referred to as optico-spinal MS; although recognised as a distinct disease in Western countries, it was often misdiagnosed as relapsing-remitting MS due to its rarity. However, in 2004 a disease-specific autoantibody was discovered in the sera of patients with NMO (Lennon, Wingerchuk et al, 2004); this was termed NMO-IgG. A year later the target antigen of NMO-IgG was identified as the water-channel aquaporin-4 (AQP4), expressed predominantly on astrocytes (Lennon, Kryzer et al, 2005) and NMO-IgG became known as AQP4 antibodies (AQP4-Ab).

Although auto-antibodies in neurological diseases have been identified as far back as the 1970s when acetylcholine receptor antibodies were discovered in the sera of patients with myasthenia gravis (MG) (Appel, Almon et al, 1975), in the last decade there has been an explosion in the discovery of novel antigen targets in neurological disease, particularly disorders of the CNS. As well as NMO, autoantibodies have been identified in autoimmune encephalitis (Vincent, Buckley

et al, 2004; Dalmau, Tüzün et al, 2007), progressive encephalomyelitis with rigidity and myoclonus (Hutchinson, Waters et al, 2008), Morvan's syndrome (Barber, Anderson et al, 2000) and acute disseminated encephalomyelitis (ADEM) (O'Connor, McLaughlin et al, 2007). Most autoantibodies in CNS disorders are directed against neuronal antigens (Figure 1.1). Although antibodies to myelin proteins have been studied extensively in CNS demyelinating disorders, many myelin antigens are not readily accessible to extracellular antibodies, and thus antibodies against such antigens are of questionable pathological relevance. The notable exception is myelin oligodendrocyte glycoprotein (MOG), which although only a minor component of myelin, is located on the extracellular surface of the myelin sheath. MOG antibodies (MOG-Ab) have recently been identified in paediatric demyelinating disorders, particularly ADEM (O'Connor, McLaughlin et al, 2007; Brilot, Dale et al, 2009; Selter, Brilot et al, 2010; Lalive, Hausler et al, 2011; DiPauli, Mader et al, 2011). To date, AQP4 is the only astrocytic antigen implicated in neurological disease.

The discovery of auto-antibodies in neurological diseases is important since these disorders are often immuno-responsive, and the presence of a biomarker enables their rapid identification and initiation of treatment. The presence of AQP4-Ab in NMO patients has enabled clinical and pathological distinction of NMO from MS, accurate identification of new cases, and early initiation of immunosuppressive treatment.

1.1.1 NMO diagnostic criteria

NMO is a clinical diagnosis that requires the presence of both ON and TM, though not necessarily together. The widely used Wingerchuk diagnostic criteria (Wingerchuk, Hogancamp et al, 1999) originally also required absence of clinical or radiological evidence of brain disease, in an attempt to differentiate the condition from MS (Table 1.1). This is important since treatment of the two conditions is different. However, the diagnostic criteria were subsequently revised (Wingerchuk, Lennon et al, 2006) to reflect two updates in the understanding of NMO. Firstly, NMO-IgG seropositivity, now known to occur in the majority of patients, was included in the criteria, though

The diagram originally presented here cannot be made freely available via ORA because of copyright reasons.

Figure 1.1. Target antigens in autoimmune disorders of the central nervous system. The astrocytic antigen aquaporin-4 (AQP4) is the target in neuromyelitis optica (NMO) and NMO spectrum disorders (NMOSD). Neuronal antigen targets include the voltage-gated potassium channel complex proteins leucine-rich, glioma-inactivated 1 (LGI1) and contactin associated protein-2 (CASPR-2), together with N-methyl D-aspartate receptor (NMDAR), gamma-aminobutyric acid B receptor ($GABA_BR$) and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptor (AMPAR); antibodies to these antigens are found in autoimmune encephalitis. LGI1 and CASPR-2 antibodies are also found in patients with Morvan's syndrome. Glycine receptor (GlyR) antibodies have been described in patients with progressive encephalomyelitis with rigidity and myoclonus (PERM). Antibodies to the myelin antigen myelin oligodendrocyte glycoprotein (MOG) have been found mainly in acute disseminated encephalomyelitis (ADEM).

Table 1.1. Original and revised Wingerchuk diagnostic criteria for NMO

Wingerchuk 1999 diagnostic criteria for NMO		Wingerchuk 2006 diagnostic criteria for NMO
Optic neuritis **AND** Myelitis **AND** No evidence of clinical disease outside the optic nerves or spinal cord **AND at least 1 of:** Brain MRI not meeting Paty criteria for MS Spinal cord lesion on MRI extending over ≥3 vertebral segments CSF pleocytosis >50 WBC/mm^3 or >5 neutrophils/mm^3 **OR at least 2 of:** Bilateral optic neuritis Severe optic neuritis with fixed visual acuity of <20/200 in at least one eye Severe, fixed, attack related weakness in at least one limb		Optic neuritis **AND** Myelitis **PLUS at least 2 of:** Spinal cord lesion on MRI extending over ≥3 vertebral segments Brain MRI not meeting Paty criteria for MS NMO-IgG seropositive status

NMO = neuromyelitis optica; MRI = magnetic resonance imaging; MS = multiple sclerosis; CSF = cerebrospinal fluid; WBC = white blood cells.

it is not a prerequisite. Secondly, since the discovery of NMO-IgG, it has become recognised that brain disease does occur in NMO, and patients with brain disease can now be included, providing their MRI scan is not 'MS-like'.

Diagnosis of NMO by the widely used 2006 Wingerchuk revised criteria requires both ON and TM and at least two of three supportive criteria: LETM on MRI, brain MRI not fulfilling diagnostic criteria for MS, and NMO-IgG positivity (Table 1.1).

1.1.2 The broadening phenotype of NMO – NMO spectrum disorders

AQP4-Ab are highly specific for NMO/NMOSD and it is extremely rare to find them in healthy individuals or in patients with other neurological conditions (Takahashi, Fujihara et al, 2007; Waters, Jarius et al, 2008; Waters, McKeon et al, 2012). Thus, the discovery of AQP4-Ab has inevitably led to broadening of the NMO clinical phenotype. It is now recognised that some patients have limited forms of the disease such as monophasic or recurrent LETM or severe bilateral or recurrent ON (Cabrera-Gomez, Bonnan et al, 2009; Bizzoco, Lolli et al, 2009). Patients with these limited phenotypes are said to have NMO spectrum disorders (NMOSD) (Wingerchuk, Pittock et al, 2007). The current diagnostic criteria (Wingerchuk, Lennon et al, 2006) do not include patients with limited phenotypes i.e. NMOSD, even if AQP4-Ab positive; however most specialists agree that they should be treated the same as those with the full NMO phenotype, with early and aggressive immunosuppression, and the modern meaning of the term NMO generally includes these patients.

1.2 Clinical and radiological features of NMO

1.2.1 Demographic features of NMO

Previous clinical NMO studies have largely been published prior to the availability of AQP4-Ab testing (O'Riordan, Gallagher et al, 1996; Wingerchuk, Hogancamp et al, 1999; Cabre, Heinzlef et al, 2001; de Seze, Stojkovic et al, 2002; Papais-Alvarenga, Miranda-Santos et al, 2002; Ghezzi, Bergamaschi et al, 2004) or included AQP4-Ab seronegative patients in their analyses (Nakashima, Fujihara et al, 2006; Merle, Olindo et al, 2007; Rivera, Kurtzke et al, 2008; Bichuetti, Oliveira et

al, 2009; Bizzoco, Lolli et al, 2009; Cabre, González-Quevedo et al, 2009; Cabrera-Gomez, Bonnan et al, 2009; Cabrera-Gomez, Kurtzke et al 2009; Adoni, Lino et al, 2010; Collongues, Marignier et al, 2010; Sahraian, Moinfar et al, 2010; Akman-Demir, Tüzün et al, 2011). Additionally, some excluded patients with brain involvement (Papais-Alvarenga, Miranda-Santos et al, 2002), those with monophasic disease (Bichuetti, Oliveira et al, 2009; Cabre, González-Quevedo et al, 2009; Cabrera-Gomez, Bonnan et al, 2009; Adoni, Lino et al, 2010; Sahraian, Moinfar et al, 2010) or those with limited forms of NMO (de Seze, Stojkovic et al, 2002; Papais-Alvarenga, Miranda-Santos et al, 2002; Ghezzi, Bergamaschi et al, 2004; Merle, Olindo et al, 2007; Bichuetti, Oliveira et al, 2009; Cabre, González-Quevedo et al, 2009; Cabrera-Gomez, Bonnan et al, 2009; Adoni, Lino et al, 2010; Sahraian, Moinfar et al, 2010; Akman-Demir, Tüzün et al, 2011) and thus provided information on the clinical syndrome of NMO as defined by the Wingerchuk criteria (Wingerchuk, Hogancamp et al, 1999; Wingerchuk, Lennon et al, 2006), rather than the expanded spectrum of NMO associated with AQP4-Ab.

Such studies have shown that NMO has a marked female predominance ranging from 3:1 to 10:1 and can occur at any age from childhood to old age, with a mean onset age of 30 to 40 years (Wingerchuk, Hogancamp et al, 1999; Cabre, Heinzlef et al, 2001; de Seze, Stojkovic et al, 2002; Papais-Alvarenga, Miranda-Santos et al, 2002; Ghezzi, Bergamaschi et al, 2004; Merle, Olindo et al, 2007; Rivera, Kurtzke et al, 2008; Bichuetti, Oliveira et al, 2009; Cabre, González-Quevedo et al, 2009; Collongues, Marignier et al, 2010; Akman-Demir, Tüzün et al, 2011). NMO is seen worldwide in patients of all ethnicities, but is over-represented in Asia and in those of Afro-Caribbean origin.

1.2.2 Clinical features of NMO

NMO is characterised by attacks of CNS inflammation that most commonly affect the optic nerves and spinal cord. Although when NMO was originally described by Devic, the occurrence of simultaneous or sequentially occurring ON and TM at onset was a hallmark, the majority of patients present with either ON or TM; only a minority will present with both together. The proportion of patients presenting with simultaneous or sequential ON/TM varies between studies,

with some showing that this presentation is rare, affecting less than 5% of NMO patients (Ghezzi, Bergamaschi et al, 2004; Cabrera-Gomez, Kurtzke et al, 2009; Sahraian, Moinfar et al, 2010) whilst others report figures of greater than 20% (de Seze, Stojkovic et al, 2002; Papais-Alvarenga, Miranda-Santos et al, 2002; Rivera, Kurtzke et al, 2008; Bichuetti, Oliveira et al, 2009; Collongues, Marignier et al, 2010). More commonly, attacks of ON and TM are separated by months or even years; the median time from ON to TM or vice versa is between 6 and 24 months (Wingerchuk, Hogancamp et al, 1999; Rivera, Kurtzke et al, 2008; Bichuetti, Oliveira et al, 2009; Cabrera-Gomez, Bonnan et al, 2009; Akman-Demir, Tüzün et al, 2010; Collongues, Marignier et al, 2010). Occasionally the first attack is preceded by infection such as varicella zoster (Heerlein, Jarius et al, 2009; Sellner, Hemmer et al, 2010) or follows vaccination (Menge, Cree et al, 2012). Pregnancy may also be the precipitant (Bourre, Marignier et al, 2012; Kim, Kim et al, 2012).

ON in NMO is typically unilateral and severe but may be bilateral. Recovery is often poor, with up to 40% of patients left blind after a single attack (Wingerchuk, Hogancamp et al, 1999; Merle, Olindo et al, 2007). Myelitis is also often severe, with limb weakness and sphincter involvement, and frequently leaves patients with severe intractable pain and spasms (Kanamori, Nakashima et al, 2011; Kim, Go et al, 2012; Qian, Lancia et al, 2012). Longitudinally extensive signal change on MRI, by definition extending over at least three vertebral segments, is a hallmark of NMO.

Until the discovery of AQP4-Ab, brain involvement was thought not to occur in NMO. However, in recent years, several characteristic clinical features due to brain and brainstem involvement in NMO have become apparent. These include intractable vomiting and/or hiccups due to inflammation in the area postrema (Apiwattanakul, Popescu et al, 2010; Riphagen, Modderman et al, 2010; Wang, Lee et al, 2011), which have been reported to occur as the first presentation in 12% of AQP4-Ab positive patients (Apiwattanakul, Popescu et al, 2010), and hypothalamic dysfunction causing hypersomnolence, hypothermia, hyperphagia, hyponatraemia and hyperprolactinaemia (Poppe, Lappiere et al, 2005; Petravić, Habek et al, 2006; Carlander, Vincent et al, 2008; Nozaki, Shimohata et al, 2008; Baba, Nakashima et al, 2009; Viegas, Weir et al, 2009; Deguchi, Kono et al, 2012; Suzuki, Nakamura et al, 2012). Additionally, hemispheric, cerebellar

and brainstem lesions can also occur causing symptoms such as hemiparesis, hemisensory loss, vertigo, ataxia, diplopia and impaired consciousness (Chan, Tse et al, 2011; Wang, Lee et al, 2011); brain involvement is relatively common in children (McKeon, Lennon et al, 2008) and in non-Caucasian populations (Chan, Tse et al, 2011; Wang, Lee et al, 2011). In fact, in Asians, brain involvement as the first attack has been reported to occur in 9 to 18% patients (Chan, Tse et al, 2011; Nagaishi, Takagi et al, 2011; Wang, Lee et al, 2011), and between one quarter and one third will have a clinical attack of brainstem involvement at some stage in their disease (Chan, Tse et al, 2011; Wang, Lee et al, 2011).

Even before NMO was recognised as an auto-antibody mediated condition, a high frequency of co-existing autoantibodies and autoimmune diseases was apparent (O'Riordan, Gallagher et al, 1996; Wingerchuk, Hogancamp et al, 1999; de Seze, Stojkovic et al, 2002; Ghezzi, Bergamaschi et al, 2004). Both organ-specific and non-organ specific autoimmune disorders may co-exist in NMO patients, including myasthenia gravis (MG), Sjögren's syndrome, systemic lupus erythematosus (SLE) and thyroid disease (Pittock, Lennon et al, 2008; Uzawa, Mori et al, 2009; Collongues, Marignier et al, 2010; Leite, Coutinho et al, 2012).

1.2.3 Disease course and prognosis in NMO

The majority of NMO patients follow a relapsing course (de Seze, Stojkovic et al, 2002; Wingerchuk, Weinshenker et al, 2003; Ghezzi, Bergamaschi et al, 2004; Rivera, Kurtzke et al, 2008; Collongues, Marignier et al, 2010; Akman-Demir, Tüzün et al, 2011). Annualised relapse rates (ARR) are between 0.4 and 1.3 (Ghezzi, Bergamaschi et al, 2004; Bichuetti, Oliveira et al, 2009; Cabre, González-Quevedo et al, 2009; Cabrera-Gomez, Kurtzke et al, 2009; Sahraian, Moinfar et al, 2010) and appear to decrease with disease duration, particularly after the first two years (Cabre, González-Quevedo et al, 2009; Collongues, Marignier et al, 2010). This may be explained by regression to the mean, treatment effects, or reduced disease activity with time.

Disability in NMO is substantial and related to relapses. A secondary progressive course, in contrast to MS, is not seen (Wingerchuk, Hogancamp et al, 1999; Matsuoka, Matsushita et al,

2007; Wingerchuk, Pittock et al, 2007). A significant proportion of patients will develop unilateral or bilateral visual impairment or require the need for a walking aid. Table 1.2 summarises studies reporting disability outcomes in NMO.

The median time to developing an Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) score of 6 (equivalent to requiring a stick to walk) has been variably reported at between three and 10 years (Ghezzi, Bergamaschi et al, 2004; Bichuetti, Oliveira et al, 2009; Cabre, González-Quevedo et al, 2009; Collongues, Marignier et al, 2010), and the median time to permanent severe unilateral and bilateral visual loss has been reported to be two to three and 11 to 15 years respectively (Merle, Olindo et al, 2007; Cabre, González-Quevedo et al, 2009). Even patients who initially appear to have a relatively benign course can go on to develop significant disability due to late disabling relapses (Collongues, Cabre et al, 2011). Mortality from NMO also occurs and figures have been reported to be as high as 33% after 9.5 years' disease duration (Merle, Olindo et al, 2007), although lower rates have been reported in more recent studies (Collongues, Marignier et al, 2010; Sahraian, Moinfar et al, 2010).

1.2.4 Radiological features of NMO

It is now clear that many NMO patients, probably even the majority at some point, have radiological evidence of brain involvement (Pittock, Lennon et al, 2006; Cabrera-Gomez, Quevedo-Sotolongo et al, 2007; Li, Xie et al, 2008; Bichuetti, Oliveira et al, 2009; Wang, Liu et al 2011; Lu, Qiu et al, 2010; Matsushita, Isobe et al 2010; Collongues, Marignier et al, 2010; Kim, Kim et al, 2011a). The most common abnormalities are multiple T2 white matter lesions that are 'non-specific' or 'MS-like', which occur in around two thirds of patients (Pittock, Lennon et al, 2006; Cabrera-Gomez, Quevedo-Sotolongo et al, 2007; Li, Xie et al, 2008; Bichuetti, Oliveira et al, 2009; Wang, Liu et al, 2011; Kim, Kim et al, 2011a; Sinnecker, Dörr et al, 2012). Although many patients fulfil radiological MS diagnostic criteria, particularly later on in the disease course (Pittock, Lennon et al, 2006; Matsuoka, Matsushita et al, 2007; Li, Xie, et al, 2008; Kim, Kim et al, 2011a; Downer, Leite et al, 2012), these lesions demonstrate differences when compared to lesions seen in MS. NMO lesions are predominantly located in the deep white matter, in contrast to MS

Table 1.2. Studies reporting disability outcomes in NMO

Study	N	Ethnicity	Disease duration	Severe unilateral visual impairment by last FU	Severe bilateral visual impairment by last FU	Motor disability by last FU
O'Riordan, Gallagher et al, 1996	12	42% Caucasian	N/A	83% unilateral or bilateral visual loss		100% unable to walk unaided
de Seze, Stojkovic et al, 2002	13	N/A	8.6 years	N/A	31%	77% unable to walk unaided
Papais-Alvarenga, Miranda-Santos et al, 2002	24	42% Caucasian 58% Mulatto	7.7 years	N/A	41%	42% unable to walk unaided 38% wheelchair bound
Merle, Olindo et al, 2007	30	Afro-Caribbean	9.7 years	20%	50%	63% unable to walk unaided
Rivera, Kurtzke et al, 2008	34	Mexican Mestizos	5.8 years	N/A	53%	N/A
Bichuetti, Oliveira et al, 2009	41	N/A	7.4 years	N/A	N/A	39% unable to walk unaided
Bizzoco, Lolli et al, 2009	13	92% Caucasian 8% Hispanic	9.8 years	38%	N/A	N/A
Cabre, González-Quevedo et al, 2009	96	French West Indies/Cuba	9.5 years	68%	48%	N/A
Collongues, Marignier et al, 2010	125	87% Caucasian	8.7 years	31%	13%	56% unable to walk unaided
Sahraian, Moinfar et al, 2010	44	All Iranian	4.7 years	30%	16%	14% unable to walk unaided 9% wheelchair bound
Nagaishi, Takagi et al, 2011	583	All Japanese	N/A	16% unilateral or bilateral blindness, 28% severe visual impairment		N/A

N = number; FU = follow up; N/A = not available.

lesions which are largely adjacent to the lateral ventricles (Sinnecker, Dörr et al, 2012). Absence of cortical lesions can also help differentiate NMO from MS (Calabrese, Oh et al, 2012; Sinnecker, Dörr et al, 2012) and in the corpus callosum, splenium lesions are more common in NMO than MS (Makino, Ito et al, 2013).

Whilst small T2 white matter lesions are the commonest radiological finding in NMO, they are not specific for the disease. However, other brain abnormalities have been identified that are highly characteristic for NMO (Pittock, Lennon et al, 2006); these occur in AQP4-rich peri-ependymal areas (Figure 1.2) (Li, Xie et al, 2008; Kim, Kim et al, 2011a; Wang, Liu et al 2011). However, whilst highly characteristic for NMO, such lesions only occur in a minority (5 to 10%) of NMO patients (Wang, Liu et al, 2011; Chan, Tse et al, 2011). Other brain abnormalities seen in NMO include brainstem lesions, particularly of the centro-dorsal medulla and the pons (Lu, Qiu et al, 2010; Kim, Kim et al, 2011a), and large, sometimes tumefactive, hemispheric lesions (Matsushita, Isobe et al, 2010), which are seen relatively commonly in children (McKeon, Lennon et al, 2008) and in non-Caucasian patients (Nakamura, Misu et al, 2009; Kim, Kim et al, 2011a).

LETM, defined as T2 hyperintensity on spinal MRI extending over ≥3 vertebral segments, has long been recognised as a hallmark of NMO, and is incorporated into the diagnostic criteria (Wingerchuk, Lennon et al, 2006). The fact that the majority of NMO patients have such lesions has been confirmed in many studies (O'Riordan, Gallagher et al, 1996; Wingerchuk, Hogancamp et al, 1999; de Seze, Stojkovic et al, 2002; Papais-Alvarenga, Miranda-Santos et al, 2002; Ghezzi, Bergamaschi et al, 2004; Rivera, Kurtzke et al, 2008; Bichuetti, Oliveira et al, 2009; Collongues, Marignier et al, 2010; Sahraian, Moinfar et al, 2010; Akman-Demir, Tüzün et al, 2011; Nagaishi, Takagi et al, 2011), though it is recognised that short cord lesions can also occur (Papais-Alvarenga, Miranda-Santos et al, 2002; Ghezzi, Bergamaschi et al, 2004; Matsuoka, Matsushita et al, 2007; Lu, Qiu et al, 2010). NMO cord lesions have a propensity to involve the central grey matter, with peripheral sparing (Matsuoka, Matsushita et al, 2007; Nakamura, Miyazawa et al, 2008; Krampla, Aboul-Enein et al, 2009; Yanagawa, Kawachi et al, 2009; Lu, Qiu et al, 2010; Downer, Leite et al, 2012), are often associated with cord oedema (Krampla, Aboul-Enein et al,

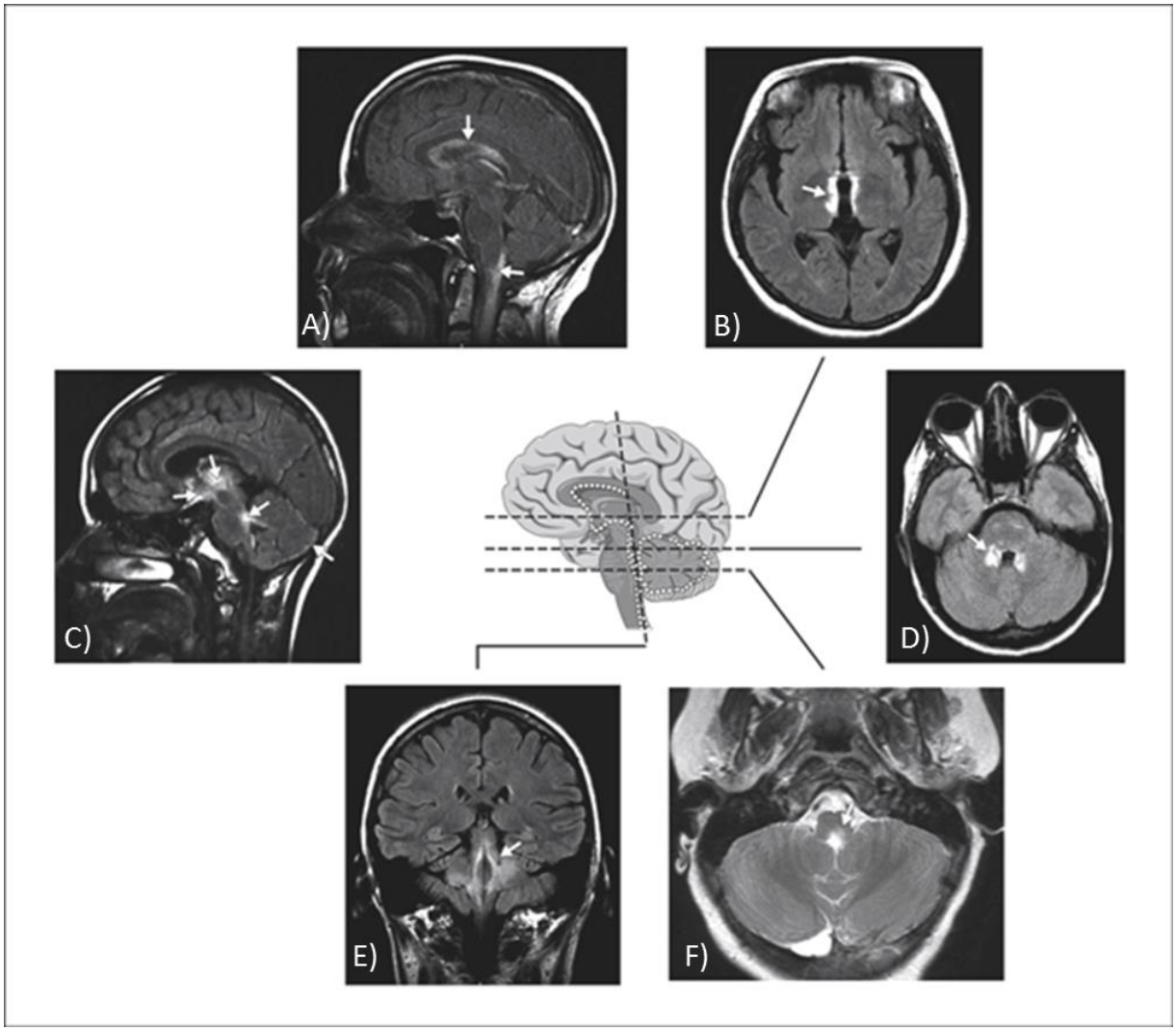


Figure 1.2. Characteristic brain MRI lesions in patients with AQP4-Ab. Brain lesions in NMO occur in peri-ependymal regions that have high AQP4 expression, including around lateral ventricles (A), the 3rd ventricle (B), thalamus and hypothalamus (C) and fourth ventricle and aqueduct (D, E, F). Adapted from Pittock, Weinshenker et al, 2006.

2009), tend to occur in the cervical and upper to mid thoracic cord (Matsuoka, Matsushita et al, 2007; Krampla, Aboul-Enein et al, 2009) and may be associated with hypointensity on T1 weighted imaging (Nakamura, Miyazawa et al, 2008; Downer, Leite et al, 2012). Following an acute attack, cord atrophy and residual T2 hyperintensity consistent with gliosis may be seen (Krampla, Aboul-Enein et al, 2009).

1.2.5 Neuropathological findings in NMO

Studies describing the typical pathological features of NMO lesions have shown perivascular deposition of immunoglobulin and complement in a distribution corresponding to AQP4 expression (Lucchinetti, Mandler et al, 2002; Misu, Fujihara et al, 2006; Misu, Fujihara et al, 2007; Roemer, Parisi et al, 2007; Yanagawa, Kawachi, et al 2009; Parratt & Prineas, 2010), infiltration of granulocytes and eosinophils (Mandler, Davis et al, 1993; Lucchinetti, Mandler et al, 2002; Roemer, Parisi et al, 2007), thickening and hyalinization of blood vessel walls (Lucchinetti, Mandler et al, 2002; Misu, Fujihara et al, 2007; Roemer, Parisi et al, 2007; Yanagawa, Kawachi, et al 2009), demyelination (Lucchinetti, Mandler et al, 2002; Yanagawa, Kawachi, et al 2009; Parratt & Prineas 2010), cavitation and necrosis (Lucchinetti, Mandler et al, 2002; Misu, Fujihara et al, 2006; Misu, Fujihara et al, 2007) and loss of AQP4 and the astrocyte marker glial fibrillary acidic protein (GFAP) within lesions i.e. astrocyte loss (Misu, Fujihara et al, 2006; Misu, Fujihara et al, 2007; Roemer, Parisi et al, 2007; Yanagawa, Kawachi, et al 2009; Lee, Metz et al, 2010).

1.2.6 Treatment of NMO

Acute attacks in NMO must be treated promptly and aggressively to prevent disability. Intravenous methylprednisolone for three to five days followed by high dose oral prednisolone is the mainstay of treatment and plasma exchange is considered beneficial in severe or steroid-resistant attacks (Bonnan, Valentino et al, 2009; Magana, Keegan et al, 2011; Bonnan & Cabre, 2012).

There have been no randomised treatment trials in NMO but azathioprine, given alongside low dose corticosteroids, is used widely as first-line therapy and has been shown in several studies to

reduce relapse frequency (Bichuetti, Lobato de Oliveira et al 2010; Mandler, Ahmed et al, 1998; Costanzi, Matiello et al, 2011). Mycophenolate mofetil, methotrexate and rituximab have also been shown to reduce relapse rates in case series (Cree, Lamb et al, 2005; Capobianco, Malucchi et al, 2007; Jacob, Weinshenker et al, 2008; Jacob, Matiello et al, 2009; Kitley, Elsone et al, 2013) and are alternative agents. The aim of treatment is to abolish or at least significantly reduce relapses to prevent relapse-related disability.

Immunomodulatory agents that are used to treat MS, such as natalizumab, interferon beta, fingolimod and alemtuzumab, are ineffective in NMO and may even exacerbate relapses (Shimizu, Yokoyama et al, 2008; Palace, Leite et al, 2010; Shimizu, Hatanaka et al, 2010; Qian, Cross et al, 2011; Barnett, Prineas et al, 2012; Jacob, Hutchinson et al, 2012; Min, Kim et al, 2012). The exception is glatiramer acetate, which may show some efficacy in NMO (Bergamaschi, 2003; Gartzen, Limmroth et al, 2007).

1.3 NMO-IgG and AQP4 antibodies

1.3.1 Discovery of NMO-IgG by tissue indirect immunofluorescence

In 2004, IgG from NMO patient sera (NMO-IgG) was shown by indirect immunofluorescence (IIF) on mouse tissue cryosections to bind in the regions of the pia, subpia and abluminal face of cerebral microvessels in white and grey matter of the cerebellum, midbrain and spinal cord (Lennon, Wingerchuk et al, 2004). Binding of NMO-IgG to the distal collecting tubules in the renal medulla, and to the basolateral membranes of parietal cells in gastric mucosa suggested the water channel AQP4 as the target antigen and this was confirmed by demonstrating co-localisation of patient IgG with commercial rabbit anti-AQP4 antibody and also by demonstrating binding of patient IgG to a cell-line expressing AQP4 at the cell surface (Lennon, Kryzer et al, 2005). Binding of NMO patient IgG to mouse or rat tissue sections in the regions mentioned above was confirmed by independent laboratories and shown to be highly specific for NMO (Jarius, Franciotta et al, 2007; Waters, Jarius et al, 2008; Vincent, Saikali et al, 2008; Marignier, Nicolle et

al, 2010), although in all of these studies, a proportion of patients with clinical features of NMO were negative for NMO-IgG.

1.3.2 Development of AQP4-Ab assays

Since the original discovery of NMO-IgG by tissue IIF, numerous other assays have been developed that specifically detect binding of NMO-IgG to AQP4; thus the antibodies detected by these assays are generally referred to as AQP4-Ab whereas antibodies detected by tissue IIF are known as NMO-IgG. These assays are summarised in Table 1.3. All have excellent specificity, though sensitivities vary markedly depending on the individual assay used, and patient cohorts. Where studies have directly compared the sensitivity of multiple assays, cell-based assays (CBAs), which detect patient IgG binding to cell-lines expressing AQP4 at the cell surface (Figure 1.3), have consistently been shown to have the highest sensitivity (Takahashi, Fujihara et al, 2007; Waters, Jarius et al, 2008; Chan, Kwan et al, 2010; Waters, McKeon et al, 2012). The original tissue IIF assay misses 17 to 25% of AQP4-Ab positive cases compared to CBAs (Waters, Jarius et al, 2008; Chan, Kwan et al, 2010; Waters, McKeon et al, 2012).

1.4 Pathogenicity of AQP4-Ab – evidence for an astrocytopathy

NMO has always been considered to be a CNS demyelinating disease. AQP4 is a water channel found predominantly on astrocytes and is not found on oligodendrocytes; consequently the pathogenic relevance of AQP4-Ab was questioned. Substantial *in vitro* and *in vivo* evidence has accumulated showing that AQP4-Ab bind to AQP4 on the surface of astrocytes and are capable of causing significant astrocyte damage and destruction; thus NMO appears to be an astrocytopathy. There is also evidence that demyelination and oligodendrocyte loss occur secondary to astrocyte damage, and mechanisms by which this may occur have been proposed.

1.4.1 *In vitro* astrocytic effects of AQP4-Ab

Human NMO-IgG binds to the extracellular domain of AQP4 *in vitro* on AQP4-transfected cells (Hinson, Pittock et al, 2007; Ratelade, Bennett et al, 2011b), and this leads to downregulation of AQP4 from the cell membrane (Hinson, Pittock et al, 2007; Hinson, Roemer et al, 2008; Vincent,

Table 1.3. Assays used for AQP4-Ab detection

Assay	General principles	Sensitivity*	References
Tissue indirect immunofluorescence (IIF)	Visualisation of characteristic binding pattern of IgG in NMO patient sera to cryosections of animal brain and spinal cord tissue	46-73%	Lennon, Wingerchuk et al, 2004; Jarius, Franciotta et al, 2007; Waters, Jarius et al, 2008; Vincent, Saikali et al, 2008; Fazio, Malosio et al, 2009; Chan, Kwan et al, 2010; Marignier, Nicolle et al, 2010; Jarius, Franciotta et al, 2012; Waters, McKeon et al, 2012
Fluorescence immunoprecipitation (FIPA)	AQP4 tagged with a fluorescent protein and incubated with patient sera. Resulting antibody-antigen complexes precipitated from solution and quantified with a fluorescence plate reader	33-76%	Waters, Jarius et al, 2008; McKeon, Fryer et al, 2009
Radio immunoprecipitation (RIPA)	Radio-labelled AQP4 incubated with patient sera. Resulting antibody-antigen complexes precipitated from solution and quantified with a radioisotope counter	33%	Fazio, Malosio et al, 2009
Enzyme-linked immunosorbent assay (ELISA)	Wells in a plate coated with AQP4 and incubated with patient sera and a fluorescent anti-human commercial antibody. Human IgG binding detected and quantified with a plate reader	46-76%	Hayakawa, Mori et al, 2008; Kim, Lee et al, 2012; Jarius, Franciotta et al, 2012; Waters, McKeon et al, 2012
Cell-based assay (CBA)	Cell-lines transfected to express AQP4 at the cell surface. Cells then incubated with patient sera and a fluorescent anti-human commercial antibody to allow visualisation of human IgG binding using a fluorescence microscope	39-91%	Takahashi, Fujihara et al, 2007; Waters, Jarius et al, 2008; Chan, Kwan et al, 2010; Waters, McKeon et al, 2012
Fluorescence-activated cell sorting (FACS)	As per CBA except human IgG binding detected and quantified using flow cytometry	30-71%	Fazio, Malosio et al, 2009; Waters, McKeon et al, 2012

*sensitivity in patients fulfilling Wingerchuk 2006 NMO diagnostic criteria.

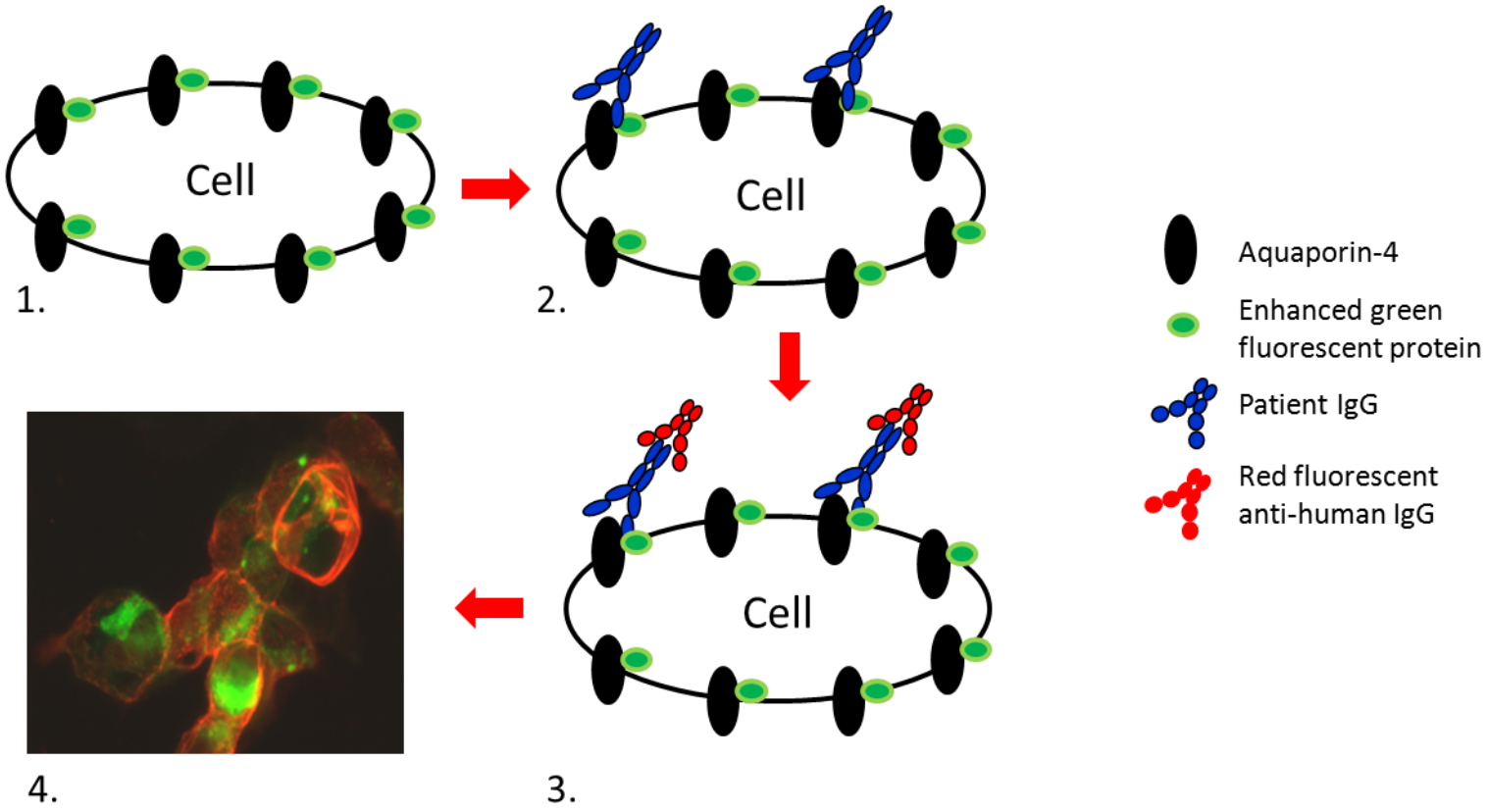


Figure 1.3. Schematic diagram of a cell-based assay used to detect AQP4-Ab. 1. Cell-lines are transfected with AQP4, together with enhanced green fluorescent protein so that transfected cells appear green. 2. AQP4-transfected cells are incubated with patient sera and AQP4-specific patient IgG binds to AQP4 at the cell-surface. 3. Cells are incubated with a red fluorescent anti-human IgG commercial antibody, which binds to patient IgG at the cell-surface. 4. A fluorescence microscope is used to visualise human IgG binding (seen in red) to AQP4-transfected cells (green). Orange represents co-localisation of human IgG and AQP4 at the cell surface.

Saikali et al, 2008; Marignier, Nicolle et al, 2010; Ratelade, Bennett et al, 2011b), with a concurrent increase in intracellular staining of AQP4 suggesting that it is internalised (Hinson, Pittock et al, 2007; Hinson, Roemer et al, 2008; Vincent, Saikali et al, 2008); this probably occurs by endocytosis as AQP4 has been shown to co-localise with cytoplasmic endosomal vesicles (Hinson, Pittock et al, 2007; Ratelade, Bennett et al, 2011b). Downregulation of AQP4 from the cell membrane is reversible following removal of NMO sera (Hinson, Pittock et al, 2007). NMO-IgG also binds to AQP4 on astrocytes in primary culture established from rodents (Hinson, Roemer et al, 2008; Sabater, Giralt et al, 2009; Marignier, Nicolle et al, 2010) and humans (Vincent, Saikali et al, 2008). However, whether this results in internalisation of AQP4 is debatable, with some studies showing limited or no internalisation of AQP4 (Ratelade, Bennett et al, 2011; Hinson, Romero et al, 2012) and others showing that internalisation does occur (Vincent, Saikali et al, 2008; Marignier, Nicolle et al, 2010).

AQP4-Ab are predominantly of the IgG1 subtype (Waters, Jarius et al, 2008; Kalluri, Illes et al, 2010) and can activate complement on the cell-membranes of AQP4-expressing cells (Hinson, Pittock et al, 2007; Waters, Jarius et al, 2008; Saadoun, Waters et al, 2010; Ratelade, Zhang et al, 2012) and astrocytes in primary culture (Kinoshita, Nakatsuji et al, 2009a; Ratelade, Zhang et al, 2012).

Exposure of astrocytes or AQP4-transfected cells to NMO-IgG *in vitro* leads to cell death (Hinson, Roemer et al, 2008; Vincent, Saikali et al, 2008; Kinoshita, Nakatsuji et al, 2009a; Sabater, Giralt et al, 2009; Kinoshita, Nakatsuji, et al, 2010), probably by necrosis (Hinson, Roemer et al, 2008; Kinoshita, Nakatsuji et al, 2009a; Sabater, Giralt et al, 2009). However, cell death only occurs in the presence of active complement (Hinson, Roemer et al, 2008; Kinoshita, Nakatsuji et al, 2009a; Sabater, Giralt et al, 2009; Saadoun, Waters et al, 2010; Ratelade, Zhang et al, 2012) or effector cells (human natural killer (NK) cells) (Vincent, Saikali et al, 2008; Ratelade, Zhang et al, 2012). Incubation of AQP4-knockout astrocytes (Kinoshita, Nakatsuji et al, 2009a) or vector-transfected cell-lines (Hinson, Pittock et al, 2007) with NMO-IgG does not result in cell death. Incubation of astrocytes or AQP4-transfected cells with NMO-IgG in the absence of complement or NK cells

leads to internalisation of AQP4 but not cell death (Hinson, Roemer et al, 2008; Marignier, Nicolle et al, 2010).

In vitro, astrocytes are less sensitive to complement-mediated NMO-IgG toxicity than AQP4-expressing cell-lines (Ratelade, Zhang et al, 2012), possibly because they express complement inhibitor proteins.

1.4.2 *In vivo* effects of AQP4-Ab

When NMO-IgG is passively transferred to rats with experimental autoimmune encephalomyelitis (EAE; an animal model of CNS inflammatory demyelinating disease), it causes worsening of the clinical severity (Bradl, Misu et al, 2009; Kinoshita, Nakatsuji, et al 2009b) and reproduces pathological changes similar to those observed in human NMO lesions (Bennett, Lam et al 2009; Bradl, Misu et al, 2009; Kinoshita, Nakatsuji, et al 2009b; Sharma, Fischer et al, 2010). Perivascular deposition of immunoglobulin and complement occur (Bennett, Lam et al 2009; Kinoshita, Nakatsuji, et al 2009b; Kinoshita, Nakatsuji, et al, 2010; Saadoun, Waters et al, 2010; Sharma, Fischer et al, 2010) in association with perivascular loss of AQP4 and GFAP immunoreactivity (Bradl, Misu et al, 2009; Kinoshita, Nakatsuji, et al 2009b; Kinoshita, Nakatsuji, et al, 2010; Saadoun, Waters et al, 2010; Sharma, Fischer et al, 2010), with appearance of AQP4 within astrocyte cytoplasm, suggesting that AQP4 is internalised (Kinoshita, Nakatsuji, et al, 2010). There is perivascular swelling of astrocytes (Bradl, Misu et al, 2009; Kinoshita, Nakatsuji, et al 2009b; Kinoshita, Nakatsuji, et al, 2010), retraction or destruction of perivascular astrocytic foot processes at the glia limitans (Sharma, Fischer et al, 2010), structural disturbance of the glia limitans (Sharma, Fischer et al, 2010) and astrocyte death (Bennett, Lam et al 2009; Bradl, Misu et al, 2009; Kinoshita, Nakatsuji, et al 2009b; Kinoshita, Nakatsuji, et al, 2010; Saadoun, Waters et al, 2010), which may occur by necrosis (Bradl, Misu et al, 2009) or by apoptosis (Kinoshita, Nakatsuji, et al, 2010). Astrocyte cytotoxicity *in vivo* appears to be dependent on the presence of non-specific inflammation, induced by co-injection with either complete Freund's adjuvant (Kinoshita, Nakatsuji, et al, 2010), human complement (Saadoun, Waters et al, 2010) or myelin basic protein (MBP)-specific T cells (Sharma, Fischer et al, 2010). NMO-like lesions do not occur

if human NMO-IgG is injected peripherally into normal animals (Bennett, Lam et al 2009; Bradl, Misu et al, 2009).

More recently, it has been shown that direct intracerebral injection of NMO-IgG can induce NMO-like lesions in normal mice but this only occurs with the addition of human complement (Saadoun, Waters et al, 2010; Ratelade, Zhang et al, 2012) or NK cells (Ratelade, Zhang et al, 2012). Thus, complement appears to be important in the pathogenesis of NMO.

1.4.3 Biomarkers of astrocyte damage in NMO

Several studies have shown that biomarkers of astrocyte damage are found in the CSF of NMO patients (Takano, Misu et al, 2008; Misu, Takano et al, 2009; Takano, Misu et al, 2010; Petzold, Marignier et al, 2011), providing further evidence for the role of astrocyte death in the pathogenesis of NMO.

GFAP is an astrocyte-specific protein that is found in the cytoplasm of astrocytes and is released after astrocyte cell death. GFAP is grossly elevated (approximately 10 000-fold) in the CSF of AQP4-Ab positive NMO patients during relapses (Takano, Misu et al, 2008; Misu, Takano et al, 2009; Takano, Misu et al, 2010) and levels have been found to correlate with disability (measured by EDSS) and length of spinal cord lesions (Misu, Takano et al, 2009; Takano, Misu et al, 2010). GFAP levels drop to normal or near normal after steroid treatment (Takano, Misu et al, 2008; Misu, Takano et al, 2009).

CSF levels of s100b, another astrocyte marker, are also markedly elevated in acute relapses of NMO (Misu, Takano et al, 2009; Takano, Misu et al, 2010; Petzold, Marignier et al, 2011), correlate with the length of spinal cord lesions (Misu, Takano et al, 2009) and decline with steroid treatment (Misu, Takano et al, 2009; Takano, Misu et al, 2010) but are less specific than GFAP levels.

CSF MBP, a marker of demyelination, is elevated in acute NMO but to a much lesser degree than CSF GFAP (Takano, Misu et al, 2010), supporting astrocyte damage being the predominant pathology in NMO.

1.4.4 Demyelination and oligodendrocyte death in NMO

In vitro, incubation of mixed astrocyte-oligodendrocyte cultures with NMO-IgG leads to a reduction in the number of both oligodendrocytes and astrocytes (Marignier, Nicolle et al, 2010). However, NMO-IgG has no effect on oligodendrocyte numbers when cultured in the absence of astrocytes, (Marignier, Nicolle et al, 2010) indicating that NMO-IgG-induced oligodendrocyte death is dependent on astrocytes. Active caspase-3 has been detected in oligodendrocyte cultures incubated with NMO-IgG, indicating that they may die by apoptosis (Marignier, Nicolle et al, 2010).

In an *ex vivo* model, where mice spinal cord sections are cultured 'live', incubation with NMO-IgG and human complement for three days was shown to result in marked loss of GFAP and AQP4, together with loss of myelin, but the latter did not occur with shorter incubation periods (Zhang, Bennett et al, 2011; Ratelade, Zhang et al, 2012), demonstrating that demyelination occurs downstream of astrocyte damage.

1.4.5 Astrocyte and oligodendrocyte interactions

Astrocytes are needed for oligodendrocyte survival. Astrocyte foot processes rich in AQP4 wrap around oligodendrocytes and the cells are linked by heterotypic gap junctions composed of connexins which facilitate transfer of metabolites and ions (Sharma, Fischer et al, 2010). The astrocyte connexins were reduced in NMO lesions *in vivo* in one study (Sharma, Fischer et al, 2010) and thus it seems plausible that AQP4-Ab-mediated astrocyte damage results in impaired functional and structural interaction between astrocytes and oligodendrocytes in NMO, which in turn leads to oligodendrocyte damage and death.

Another proposed mechanism for oligodendrocyte death in NMO is by glutamate excitotoxicity due to dysregulation of glutamate homeostasis secondary to astrocyte damage (Marignier, Nicolle et al, 2010). Glutamate is the most abundant excitatory neurotransmitter in the CNS and is also a potent neurotoxin at high doses. The most abundant glutamate transporter in the CNS, excitatory amino acid transporter-2 (EAAT2), is expressed predominantly on astrocytes and there is some

evidence that it forms a macromolecular complex with AQP4 (Hinson, Roemer et al, 2008). *In vitro*, expression of EAAT2 is markedly reduced in astrocytes from AQP4-knockout mice and this is associated with a significant reduction in the ability of these astrocytes to take up glutamate (Zeng, Sun et al, 2007). In a non-neuronal cell-line, cells transfected with AQP4 also subsequently expressed EAAT2 and this resulted in significantly increased uptake of extracellular glutamate (Hinson, Roemer et al, 2008). Incubation of astrocytes with NMO-IgG has been reported to result in downregulation of EAAT2 in addition to AQP4 in some studies (Hinson, Roemer et al, 2008; Marignier, Nicolle et al, 2010) but not others (Ratelade, Bennett et al, 2011b) and one study found that these two molecules co-localised within cytoplasmic vesicles, suggesting that they are endocytosed together (Hinson, Roemer et al, 2008). In non-pathological human CNS tissue, EAAT2 and AQP4 co-localise (Hinson, Roemer et al, 2008) and in a spinal cord lesion from an NMO patient, EAAT2 immunostaining was shown to be reduced in regions of AQP4 loss (Hinson, Roemer et al, 2008). Taken together, these findings suggest that AQP4 and EAAT2 are co-expressed on astrocyte membranes.

In vitro, glutamate uptake by astrocytes was found to be markedly reduced following incubation with NMO-IgG (Hinson, Roemer et al, 2008) although a more recent study could not confirm this (Ratelade, Bennett et al, 2011b). Incubation of astrocytes with NMO-IgG has also been shown to reduce activity of glutamine synthetase, a key enzyme in glutamate homeostasis, and extracellular glutamate levels rise in glial cell cultures incubated with NMO-IgG (Marignier, Nicolle et al, 2010). Oligodendrocytes are vulnerable to glutamate in a dose dependent manner, which may induce oligodendrocyte apoptosis (Xu, Liu et al, 2008) and glutamate also sensitises oligodendrocytes to complement attack (Alberdi, Sanchez-Gomez et al, 2006). Blockade of EAAT2 glutamate receptors on astrocytes, which would presumably have the same effect as the EAAT2 receptor downregulation reported in NMO, has been shown to induce massive demyelination and axonal loss in optic nerves (Domercq, Etxebarria et al, 2005). Thus, glutamate-mediated excitotoxicity as a consequence of astrocyte damage may be a mechanism of secondary oligodendrocyte death in NMO.

1.4.6 AQP4-Ab and relation to disease activity

It is now generally accepted that the presence of AQP4-Ab in patient sera is pathologically and clinically relevant and it is known that AQP4-Ab seropositivity predicts a high risk of relapsing disease (Weinshenker, Wingerchuk et al, 2006; Matiello, Lennon et al, 2008; Collongues, Marignier et al, 2011). Whether AQP4-Ab levels are of clinical or prognostic significance is less clear. Table 1.4 summarises studies that have reported the clinical and prognostic significance of AQP4-Ab titres.

Several studies, using various methods of AQP4-Ab detection including FIPA scores (Jarius, Aboul-Enein et al 2008; Waters, Jarius et al, 2008), CBA scores (Waters, Jarius et al, 2008), and CBA end-point dilutions (Jarius, Franciotta et al, 2010) have shown AQP4-Ab titres to be higher during relapse than in remission in group analyses. However, three recent studies that measured ELISA scores (Kim, Kim et al, 2011b; Kim, Lee et al, 2012; Isobe, Yonekawa et al, 2013) found that within individual patients, although ELISA scores during relapses were higher than previous remission values in the majority of cases, not all relapses were associated with a rise in AQP4-Ab and high levels were not always associated with a clinical relapse.

In one study (Jarius, Aboul-Enein et al, 2008) that retrospectively tested serum samples in a small number of patients (n=5), around a 20% increase in AQP4-Ab levels per week in the 100 days prior to a relapse was estimated to occur. However, a significant rise in antibody titres from trough remission levels did not necessarily herald a clinical relapse and AQP4-Ab levels could even be higher than that documented during a previous relapse without resulting in relapse. In other words, there was no general threshold level across patients associated with clinical relapse. However, in a later study by the same group (Jarius, Franciotta et al, 2010), CBA end-point dilution titres >1:250 were associated with relapses in all cases and titres <1:250 with disease quiescence.

Two studies have looked at a potential correlation of AQP4-Ab titres with MRI findings. The first (Takahashi, Fujihara et al, 2007) found a significant correlation between spinal MRI lesion length in patients with LETM and serum antibody titres. However, this correlation was not confirmed in

Table 1.4. Studies reporting clinical and prognostic significance of AQP4-Ab titres

Study	N	Assay	Relapse vs. remission titres	Titres and clinical/radiological features
Hayakawa, Mori et al, 2008	9 to 20 for each analysis	ELISA scores	Trend towards higher titres during relapse	Trend towards correlation between EDSS scores during relapse and titres
Kim, Lee et al, 2012	51	ELISA scores	Relapses usually, though not always, associated with a rise in titres	N/A
Takahashi, Fujihara et al, 2007	35	End-point dilution on M1 CBA	N/A	Higher titres in blind patients than in non-blind patients. Nadir MRI lesion length correlated with nadir titres.
Hinson, McKeon et al, 2009	12	End-point dilution on tissue IIF	N/A	Titres did not differ between those with severe attacks (n=6) and those with mild attacks (n=6)
Waters, Jarius et al, 2008	25; earliest available sample used	FIPA scores	FIPA scores higher during relapse than remission	No correlation between FIPA scores and MRI lesion length
Jarius, Aboul-Enein et al, 2008	8	FIPA scores	FIPA scores higher during relapse than remission in paired samples	N/A
Jarius, Franciotta et al, 2010	N/A	End-point dilution on CBA	Median relapse titres (n=20) higher than remission (n=6)	No difference in titres between TM (n=13) and ON (n=6) attacks
Kim, Kim et al, 2011b	21	ELISA scores	13 of 14 relapses associated with sustained high titres or rising titres but levels at relapse varied between and within individuals and rising titres did not always lead to relapse	
Isobe, Yonekawa et al, 2013	38 total; 12 serial	ELISA scores using purified M1	No difference between relapse and remission samples	Patients with a history of ON had higher titres than those without ON; patients with severe ON had lower titres than patients with mild ON

N = number; ELISA = enzyme-linked immunosorbence assay; CBA = cell-based assay; IIF = indirect immunofluorescence; FIPA = fluorescence immunoprecipitation; EDSS = Expanded Disability Status Scale; MRI = magnetic resonance imaging; TM = transverse myelitis; ON = optic neuritis.

a later study (Waters, Jarius et al, 2008). The study by Takahashi and colleagues (Takahashi, Fujihara et al, 2007) also looked at a correlation between antibody titres and brain MRI findings and found that four patients with large or extensive brain lesions had high antibody titres whereas those with no brain lesions had low titres. However, no details concerning disease duration, disease activity or treatment are mentioned in this study.

A few studies have looked at whether AQP4-Ab levels are of prognostic significance. The median AQP4-Ab titres during myelitis attacks were shown to be similar to median AQP4-Ab titres during ON attacks but a sample taken from one patient with simultaneous ON and TM had relatively high titres (Jarius, Franciotta et al, 2010). Two studies (Takahashi, Fujihara et al, 2007; Mader, Lutterotti et al, 2010) have found AQP4-Ab levels in patients with a full NMO phenotype to be higher than in those with limited phenotypes i.e. NMOSD, and in patients with recurrent ON higher IIF end-point dilution titres were found in those who subsequently went on to have TM compared to those whose disease remained restricted to the optic nerves (Matiello, Lennon et al, 2008). However, for some of these studies, it is unclear whether samples were taken during relapse or remission, on or off treatment and at what stage in the disease, which may confound outcomes.

One study (Takahashi, Fujihara et al 2007) found significantly higher CBA AQP4-Ab titres in patients who were blind from ON compared to those who had had ON but weren't blind but they do not comment on disease duration, number of ON attacks, disease activity or treatment details and it is not clear at what stage samples were taken. No correlation has been found between AQP4-Ab titres and EDSS scores (Takahashi, Fujihara et al, 2007; Hayakawa, Mori et al, 2008; Hinson, McKeon et al, 2009) or ARR (Takahashi, Fujihara et al, 2007). However, it has been shown that the degree of complement-mediated lysis is significantly greater in patients with severe attacks than those with mild attacks (Hinson, McKeon et al, 2009), which led this group to suggest that it was the ability of AQP4-Ab to activate complement, rather than the amount of antibodies *per se* that influenced attack severity.

1.5 AQP4 – structure, function and distribution

1.5.1 AQP4 expression in the nervous system and other tissues

AQP4 is the most abundant water channel in the CNS and it is believed to enable bidirectional transport of water across the blood-brain interface (Amiry-Moghaddam, Otsuka et al, 2003). Within the CNS, AQP4 expression is largely restricted to astrocytes (Nielsen, Nagelhus et al, 1997; Venero, Vizuete et al, 1999), in which it is predominantly expressed on the cell membrane of the perivascular foot processes that interact functionally and structurally with capillary endothelial cells to make up the blood brain barrier (Nielsen, Nagelhus et al, 1997; Rash, Yasumura et al, 1998; Nicchia, Nico et al, 2004; Tait, Saadoun et al, 2008).

Although AQP4 messenger ribonucleic acid (mRNA) is widely distributed throughout the CNS (Jung, Bhat et al, 1994), at a protein level it is particularly prevalent in periventricular areas (Venero, Vizuete et al, 1999), especially around the floor of the 4th ventricle (Roemer, Parisi et al, 2007), and at the glia limitans externa where the pial surface of the brain is in contact with cerebrospinal fluid (CSF) in the subarachnoid space (Jung, Bhat et al, 1994; Nielsen, Nagelhus et al, 1997; Venero, Vizuete et al, 1999). High levels are also found in the cerebellum (Nielsen, Nagelhus et al, 1997; Neeley, Christensen et al, 1999) and within the white matter of the brainstem (Roemer, Parisi et al, 2007), whilst lower levels are seen in the cerebral cortex (Nielsen, Nagelhus et al, 1997; Venero, Vizuete et al, 1999), at the grey-white interface (Roemer, Parisi et al, 2007) and in cerebral white matter where expression is largely limited to the surface of penetrating blood vessels (Roemer, Parisi et al, 2007).

In human spinal cord (Roemer, Parisi et al, 2007; Misu, Fujihara et al, 2007), AQP4 is expressed in both white and grey matter, but is most concentrated in the central grey matter (Misu, Fujihara et al, 2007), particularly in perivascular astrocytic end feet. In the white matter, AQP4 immunostaining occurs perivascularly (Nicchia, Mastrototaro et al, 2009) in a meshwork and rim and rosette vasculocentric pattern (Roemer, Parisi et al, 2007).

AQP4 is also expressed on astrocytes in the optic nerve and in retinal Müller cells and in the plasmalemma of fast-twitch fibres in skeletal muscle (Ratelade, Bennett et al, 2011).

Extraneuronal tissues including the lungs, basolateral membrane of the kidney collecting ducts, heart, liver, basolateral membrane of tracheal surface epithelial cells, basolateral membrane of gastric parietal epithelial cells and intestines also express AQP4 (Jung, Bhat et al, 1994; Neely, Christensen et al, 1999; Saini, Fernandez et al, 2010; Ratelade, Bennett et al, 2011). However, with the exception of the lungs, these organs express low levels compared with the brain (Jung, Bhat et al, 1994; Saini, Fernandez et al, 2010).

1.5.2 AQP4-associated proteins

There is some suggestion that AQP4 may colocalise with other functionally significant proteins. These include the potassium channel Kir4.1 (Nagelhus, Horio et al, 1999; Connors & Kofuji, 2006), the glutamate transporter EAAT2 as previously mentioned (Zeng, Sun et al, 2007) and structural anchoring proteins such as α-syntrophin (Neely, Amiry-Moghaddam et al, 2001) and dystrophin, although evidence for structural or functional interaction with any of these proteins is limited.

Additionally, the heparan sulfate proteoglycan agrin may be involved in clustering of AQP4 on astrocytic membranes (Noell, Fallier-Becker et al, 2007; Fallier-Becker, Sperveslage et al, 2011; Noell, Fallier-Becker et al, 2009) as the density of AQP4 clusters is reduced in agrin-null astrocytes (Fallier-Becker, Sperveslage et al, 2011). In mice with selective knock-out of agrin in endothelial cells, AQP4 expression is significantly reduced both on immunohistochemistry and by ELISA of rat brain homogenate (Rauch, Huen et al, 2011).

1.5.3 AQP4 isoforms

The AQP4 gene has been mapped to chromosome 18 at the junction of q11.2 and q12.1 (Lu, Lee et al, 1996). The gene encodes two isoforms, M1 and M23 (Jung, Bhat et al, 1994; Lu, Lee et al, 1996; Neely, Christensen et al, 1999) utilizing distinct mRNA transcripts generated by alternative splicing of exons encoding the N-terminal domain (Lu, Lee et al, 1996).

The M1 isoform is slightly larger than the M23 isoform, being 34 kDa and 323 amino acids in length compared to the 32 kDa M23 isoform which lacks a 22 amino acid N-terminus (Figure 1.4) (Jung, Bhat et al, 1994; Lu, Lee et al, 1996; Neeley, Christensen et al, 1999). There does not appear to be any difference in the ability of these isoforms to transport water (Neely, Christensen et al, 1999).

At a cellular level, mRNA levels of the two isoforms in astrocytes have been shown to be the same (Noell, Fallier-Becker et al, 2007); at a protein level in rodents the M23 isoform has been shown to predominate (Lu, Lee et al, 1996; Neely, Christensen et al, 1999), with one study suggesting that M23 and M1 are expressed in a ratio of 3:1 (Neely, Christensen et al, 1999). M1 levels were greatest in the cerebellum and spinal cord, (Neely, Christensen et al, 1999) though this may simply reflect the higher total level of AQP4 expression in these tissues.

In the human CNS, whilst M1 mRNA was found to be generally more abundant than M23 mRNA, the ratio of M1:M23 mRNA varied, being highest in the optic nerves and spinal cord, whilst expression of M23 was greater in the cortex and cerebellum (Saini, Fernandez et al, 2010); expression of these isoforms at a protein level in humans has not been characterised. Organs outside the CNS express predominantly M23 (Frigeri, Gropper et al, 1995; Neely, Christensen et al, 1999) with only trace levels of M1 (Saini, Fernandez et al, 2010). The only exception is the lung, which expresses high levels of M1 mRNA (Saini, Fernandez et al, 2010).

1.5.4 Clustering of AQP4 to form orthogonal arrays

Individual AQP4 monomers of both the M23 and the M1 isoform assemble in astrocytic cell membranes as heterotetramers (Neely, Christensen et al, 1999), which may be comprised of M1 or M23 alone or a combination of the two (Neely, Christensen et al, 1999; Crane, Bennett et al, 2009; Jin, Rossi et al, 2011). These AQP4 heterotetramers in turn aggregate together to form supramolecular assemblies of intramembrane proteins known as orthogonal arrays of particles (OAPs) (Rash, Yasamura et al, 1998). Several lines of evidence suggest that OAPs are formed predominantly by the M23 isoform. Firstly, native protein electrophoresis experiments on AQP4-

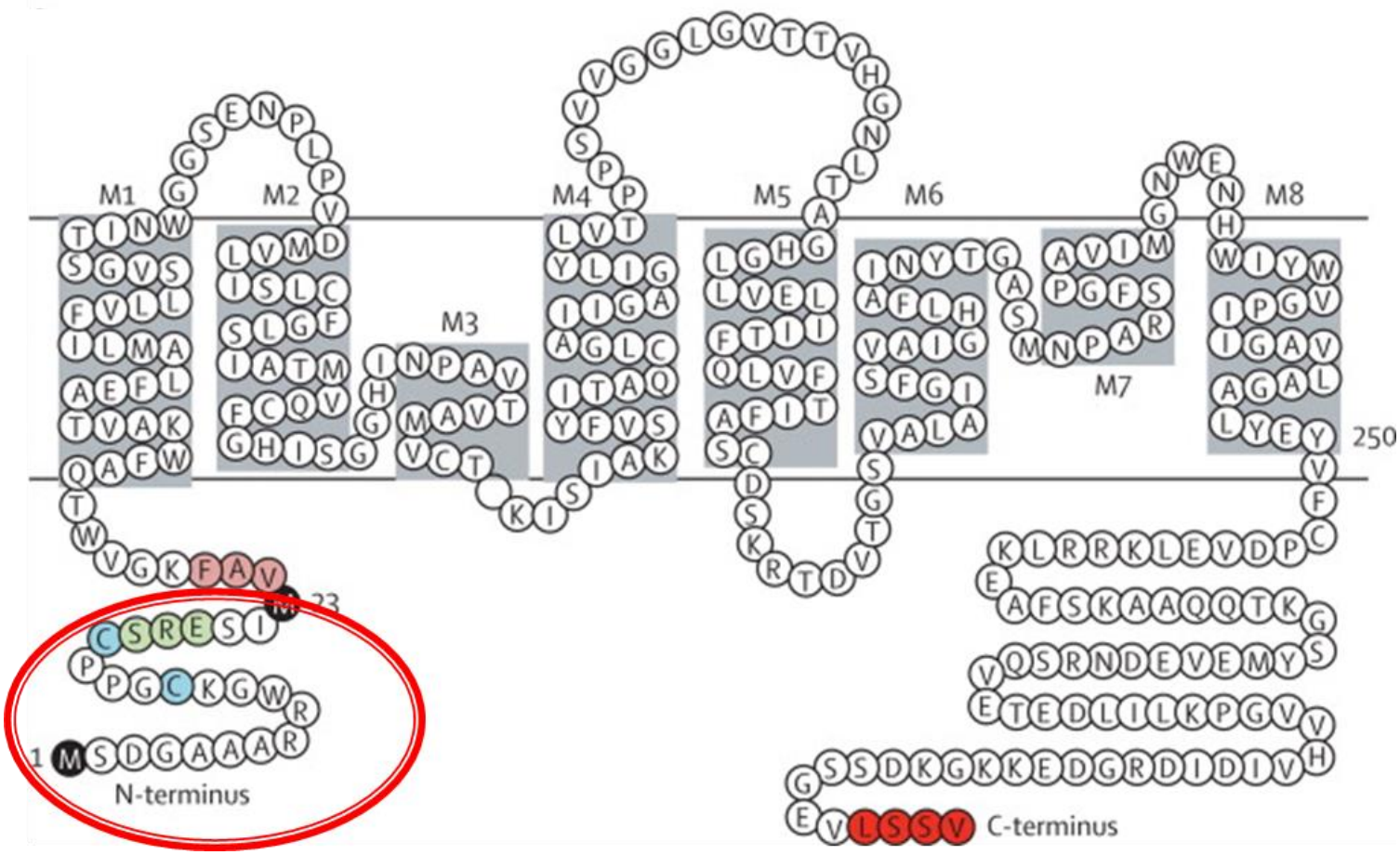


Figure 1.4. Structure of AQP4. AQP4 exists as two isoforms, a full-length M1 isoform and a shorter M23 isoform, which lacks an N-terminus (red circle). Adapted from Papadopoulos & Verkman, 2012.

transfected cells reveal multiple large bands for M23-transfected cells, whilst M1-transfected cells produce a single small band (Crane, Lam et al, 2011; Phuan, Ratelade et al, 2012). Secondly, the M23 isoform in cell membranes is relatively immobile whilst the M1 isoform diffuses freely (Crane, Bennett et al, 2009; Crane, Rossi et al, 2011). Thirdly, cells transfected with M23 have been shown, using freeze-fracture electron microscopy, to contain OAPs, which are largely absent from cells transfected with the M1 isoform (Yang, Brown et al, 1996; Furman, Gorelick-Feldman et al, 2003). However, it is believed that M1 can integrate into M23 OAPs and in doing so, reduces their size (Crane, Bennett et al, 2009; Furman, Gorelick-Feldman et al, 2003; Crane, Van Hoek et al, 2008; Jin, Rossi et al, 2011).

OAPs are believed to be formed by hydrophobic interactions between areas near the N-terminus on M23 monomers (Crane, Bennett et al, 2009; Crane & Verkman, 2009), whilst palmitoylation of N-terminal cysteine residues on M1 monomers just upstream of Met-23 is thought to interrupt OAP formation (Suzuki, Nishikawa et al, 2008; Crane, Bennett et al, 2009). This is supported by findings that mutations in the hydrophobic N-terminus of M23 prevent OAP formation in rats (Crane and Verkman, 2009) and in humans (Crane, Rossi et al, 2011), and a palmitoylation-null M1 mutant is unable to disrupt OAP formation (Crane, Bennett et al, 2009). OAP formation was recently shown to be a post-golgi phenomenon (Rossi, Baumgart et al, 2012) and may involve interaction of AQP4 with other plasma membrane proteins. As mentioned earlier, agrin may be one such protein.

1.5.5 Significance of AQP4 isoforms and OAPs in AQP4-Ab disease

Several independent groups have shown *in vitro* that whilst human NMO-IgG binds to both AQP4 isoforms, there is stronger binding to the M23 isoform (Nicchia, Mastrototaro et al, 2009; Mader, Lutterotti et al, 2010; Crane, Lam et al, 2011; Iorio, Fryer et al, 2013). Although one study suggested that NMO-IgG binds only to AQP4 in OAPs (Nicchia, Mastrototaro et al, 2009), many diagnostic AQP4 CBAs use M1-transfected cells (Hinson, Pittock et al, 2007; Takahashi, Fujihara et al, 2007; Bennett, Lam et al, 2009) and since M1 does not form OAPs, AQP4-Ab cannot only bind to OAPs.

Mader and colleagues (Mader, Lutterotti et al, 2010) found that NMO-IgG from some patients bound only to M23, some bound to both isoforms, but no patients had antibodies that bound only to the M1 isoform. Patients with both M1 and M23 antibodies had statistically significantly longer disease duration than those whose NMO-IgG bound only to M23 (Mader, Lutterotti et al, 2010) suggesting that M1 antibodies may develop later in the course of the disease.

In a recent study, relative binding of patient sera to M1 and M23 AQP4 isoforms was quantified using fluorescence ratio imaging (Crane, Lam et al, 2011). Although sera from all patients bound to both M23 and M1 isoforms and binding to M23 was stronger in all patients, there was a wide range of relative M1:M23 binding. Additionally, within a single patient, recombinant monoclonal antibodies (rAbs) generated from plasma cells in the CSF showed different relative binding to M23 and M1 (Crane, Lam et al, 2011). When a mutant M23 isoform with limited ability to form OAPs was used to transfect cells, binding affinity was less than for wild-type M23, suggesting that it is OAPs that are responsible for the enhanced binding of NMO-IgG to M23. They went on to show that Fab fragments bound equally well as whole IgG to M23, suggesting that binding occurs by monovalent as opposed to divalent binding. Thus, the authors suggested that formation of OAPs by AQP4 heterotetramers may lead to a structural change in the epitope binding site for AQP4-Ab that makes binding easier. This is supported by a recent publication from an independent group (Iorio, Fryer et al, 2013) that found that NMO-IgG from 100% of 32 strongly AQP4-Ab positive NMO/NMOSD patients bound to both M1 and M23 stably-transfected HEK cells but that only 90% of these samples bound to native M1 and M23 tetramers, whilst binding of IgG to denatured epitopes presumed to be M1 and M23 monomers was considerably less. These results suggest that a degree of NMO-IgG binding is to epitopes conformationally expressed by higher order structures (i.e. heterotetramers or OAPs) that are not found on individual AQP4 monomers.

As mentioned in section 1.4, complement-dependent cytotoxicity (CDC) appears to be a major pathogenic mechanism in NMO. It was recently shown by two independent groups (Hinson, Romero et al, 2012; Phuan, Ratelade et al, 2012) that CDC of AQP4-transfected cells occurs in cells transfected with M23 but not M1. However, the proposed explanation differed. The first

study found that M1 is rapidly internalised following exposure to NMO-IgG whilst M23 shows only partial internalisation (Hinson, Romero et al, 2012). They hypothesised that it was M23 resisting internalisation that made it sensitive to complement activation. However, the second group showed that surface expression of M23 was reduced significantly more than surface expression of M1 after exposure to NMO-IgG (Phuan, Ratelade et al, 2012). They went on to show that CDC of M23-transfected cells did not occur when the cells were transfected with an M23 mutant incapable of forming OAPs, implying that CDC is related to OAP formation. Additionally, co-transfection with M1 and M23, which reduces the size of OAPs, resulted in reduced CDC. The amount of CDC increased with increasing concentrations of either NMO-IgG or human complement. The investigators proposed that binding of the C1q complement protein to NMO-IgG was more efficient when NMO-IgG was clustered by OAPs and that the enhanced ability of M23 OAPs to activate complement is probably because of multivalent binding of C1q to multiple human NMO-IgG molecules tightly bunched on M23 OAPs whereas NMO-IgG binding to M1 heterotetramers is such that human IgGs are not close enough together for C1q to bind multivalently. Multivalent binding of C1q is required for complement activation.

Although many studies have shown that CDC occurs in NMO (Hinson, Roemer et al, 2008; Sabater, Giralt et al, 2009; Hinson, Romero et al, 2012; Phuan, Ratelade et al, 2012), antibody-dependent cell-mediated cytotoxicity (ADCC), which is mediated by degranulation of natural killer (NK) cells, also occurs (Vincent, Saikali et al, 2008; Phuan, Ratelade et al, 2012). ADCC was shown to occur in both M1 and M23 transfected cells (Phuan, Ratelade et al, 2012), suggesting that OAP formation is not necessary for ADCC.

Thus, antibodies against the M23 isoform may be more important in NMO pathogenesis for two reasons, i) that M23 is able to form OAPs, for which NMO-IgG has greater affinity and ii) OAPs are better able to activate complement.

1.6 Summary of AQP4-Ab disease

Traditionally thought of as a severe opticospinal variant of MS, NMO is now recognised as a distinct auto-antibody mediated condition. The disease-specific biomarker AQP4-Ab is found in the majority of NMO patients, and many with NMOSD. There is substantial evidence that AQP4-Ab are pathogenic, and experimental and neuropathological studies suggest that AQP4-Ab disease is an astrocytopathy, with demyelination and axonal loss occurring secondary to astrocyte damage.

AQP4-Ab are highly specific for NMO/NMOSD and because they enable differentiation of NMO from MS and other conditions with a high degree of accuracy, they have become pivotal in the investigation of patients presenting with CNS inflammation. It is now clear that the spectrum of AQP4-Ab disease is wider than first thought and although AQP4-Ab positivity has been shown to confer a high risk of relapsing disease, the clinical course and disease outcomes of patients with AQP4-Ab have not been systematically studied, and prognostic influences have not been evaluated. Additionally, AQP4 exists as two isoforms and it is unclear whether AQP4-Ab specificity to these two isoforms may have clinical significance in human disease.

1.7 AQP4-Ab seronegative NMO

Despite advances in the development of AQP4-Ab assays, a variable proportion of patients with clinical characteristics of NMO are 'seronegative', meaning that AQP4-Ab cannot be detected in their sera. These patients pose a particular problem, with diagnostic and treatment difficulties. It is unclear whether these patients represent a distinct clinical subgroup of NMO or have a condition other than NMO such as MS or ADEM. They may have a different clinical course and outcomes from patients with AQP4-Ab-mediated disease, though studies are conflicting (Nakashima, Fujihara et al, 2006; Kanzaki, Mochizuki et al, 2008; Bizzoco, Lolli et al, 2009; Cabrera-Gomez, Bonnan et al, 2009; Akman-Demir, Tüzün et al, 2011; Kiyat-Atamer et al 2013). This may in part be because most studies looking at differences between AQP4-Ab positive and negative patients used the originally described tissue IIF assay to detect AQP4-Ab, which lacks sensitivity and thus likely included false negative cases. However, even using the most sensitive assays available, a

proportion of patients remain AQP4-Ab negative (Waters, McKeon et al, 2012). Some of these patients may have autoantibodies with a different antigenic target, or their sera may contain low affinity AQP4-Ab that are below the detection limit of current assays. There have been no published studies to date specifically addressing these questions.

1.7.1 Prevalence

The prevalence of seronegative NMO varies widely and is influenced by the diagnostic criteria and assays used. In predominantly Caucasian cohorts of patients fulfilling the 2006 Wingerchuk diagnostic criteria, 23 to 50% of patients are AQP4-Ab negative (Bizzoco, Lolli et al, 2009; Collongues, Marignier et al, 2010; Kiyat-Atamer, Ekizoglu et al, 2013), whilst rates of up to 70% have been reported in non-Caucasian populations (Bichuetti, Oliveira et al, 2009; Cabre, González-Quevedo et al, 2009; Cabrera-Gomez, Kurtzke et al, 2009; Adoni, Lino et al, 2010; Sahraian, Moinfar et al, 2010), although these figures can be much less when more sensitive assays are used, with only 5% of patients fulfilling the 2006 Wingerchuk criteria being AQP4-Ab negative in a recent large Japanese study (Nagaishi, Takagi et al, 2011). This is likely in part to be related to use of different assays, but there are also likely to be genetic influences.

1.7.2 Demographic features

One striking feature of NMO is the strong female preponderance but this is less marked in some cohorts that included a large proportion of seronegative patients (Bichuetti, Oliveira et al, 2009; Collongues, Marignier et al, 2010; Sahraian, Moinfar et al, 2010) and in patients with monophasic disease who are more likely to be seronegative (Wingerchuk, Hogancamp et al, 1999; Wingerchuk, Weinshenker et al, 2003). In studies that have directly compared characteristics of seropositive and seronegative patients, a higher proportion of males in seronegative NMO has been shown in some studies, (Nagaishi, Takagi et al, 2011; Jarius, Ruprecht et al, 2012; Zhong, Wang et al, 2012) but not others (Adoni, Lino et al, 2010; Akman-Demir, Tüzün et al, 2011; Bizzoco, Lolli et al, 2009).

As mentioned earlier, the mean age at disease onset in NMO is between 30 and 40 years, but cohorts that include a large proportion of seronegative patients generally report lower figures (Cabre, González-Quevedo et al, 2009; Bichuetti, Oliveira et al, 2009; Collongues, Marignier et al, 2010; Akman-Demir, Tüzün et al, 2011; Sahraian, Moinfar et al, 2010) and onset age has also been shown to be lower in those with monophasic disease (Wingerchuk, Hogancamp et al, 1999; Wingerchuk, Weinshenker et al, 2003). In the studies that have directly compared AQP4-Ab seropositive and seronegative patients, no significant difference in age at disease onset has been found in some studies (Akman-Demir, Tüzün et al, 2011; Adoni, Lino et al, 2010; Bizzoco, Lolli et al, 2009; Jarius, Ruprecht et al, 2012; Zhong, Wang et al, 2012) though a recent large study (Nagaishi, Takagi et al, 2011) found seronegative patients to be significantly younger.

1.7.3 Clinical features

Although the original description of NMO was of monophasic simultaneous/sequential ON and myelitis (Devic, 1894), this classical 'Devic's-like' presentation is actually quite rare in AQP4-Ab positive NMO; higher rates have been reported when large proportions of seronegative patients are included in cohorts (Rivera, Kurtzke et al, 2008; Bichuetti, Oliveira et al, 2009; Akman-Demir, Tüzün et al, 2011; Collongues, Marignier et al, 2010). In studies directly comparing the onset attack type in seropositive versus seronegative patients, no difference in onset attack was found in two studies (Akman-Demir, Tüzün et al, 2011; Nagaishi, Takagi et al, 2011), though the second of these did not include ON plus TM as an option, whilst another study found a higher proportion of simultaneous ON plus TM and bilateral ON at onset in seronegative patients (Jarius, Ruprecht et al, 2012). The severity of the onset attack, measured by nadir EDSS has been found to be worse in seropositive patients in one study (Kiyat-Atamer, Ekizoglu et al, 2013).

The proportion of patients with relapsing disease was found to be no different between seropositive and seronegative patients in one study (Akman-Demir, Tüzün et al, 2011), though another found that seronegative patients were significantly more likely to have monophasic disease (Jarius, Ruprecht et al, 2012). A greater total number of relapses and higher ARR in AQP4-Ab positive patients have been found in some studies (Bizzoco, Lolli et al, 2009; Cabrera Gomez, Kurtzke et

al, 2009) but not others (Bizzoco, Lolli et al, 2009; Akman-Demir, Tüzün et al, 2011; Nagaishi, Takagi et al, 2011; Jarius, Ruprecht et al, 2012; Zhong, Wang et al, 2012; Kiyat-Atamer, Ekizoglu et al, 2013).

As mentioned previously, several characteristic clinical features of NMO have become apparent in recent years including intractable vomiting and/or hiccups, hypothalamic dysfunction and tonic spasms and pain. To date, these features have only been reported in AQP4-Ab positive patients or where AQP4-Ab status has not been determined, though no studies have systematically evaluated their presence in seronegative NMO. It is important that the frequency and severity of these features is compared between seronegative and seropositive patients to help establish whether these patients share similar disease characteristics.

NMO patients often have coexisting autoimmune disorders and/or coexisting autoantibodies. Some studies have shown similar rates of anti-nuclear antibodies (ANA) (Bizzoco, Lolli et al, 2009; Adoni, Lino et al, 2010) and antibodies to extractable nuclear antigens (ENA) (Bizzoco, Lolli et al, 2009; Adoni, Lino et al, 2010; Nagaishi, Takagi et al, 2011) and similar rates of concomitant autoimmune disorders (Nagaishi, Takagi et al, 2011) between AQP4-Ab positive and negative NMO patients, though one study did show a higher proportion of co-existing autoantibodies in seropositive patients (Pittock, Lennon et al, 2008).

1.7.4 Disability and disease outcomes

Relapse-related disability in NMO is substantial. One study has suggested that the severity of attacks is significantly worse in AQP4-Ab positive patients compared to seronegative patients (Jarius, Ruprecht et al, 2012), though recovery from attacks has been found to be similar (Akman-Demir, Tüzün et al, 2011; Jarius, Ruprecht et al, 2012). Some studies have found a higher EDSS at last follow-up (Bizzoco, Lolli et al, 2009; Cabrera Gomez, Kurtzke et al, 2009; Akman-Demir, Tüzün et al, 2011; Kiyat-Atamer, Ekizoglu et al, 2013) and a greater proportion of patients reaching EDSS 6 (Akman-Demir, Tüzün et al, 2011) in AQP4-Ab positive patients whilst others have found no differences in EDSS scores or time to EDSS endpoints (Adoni, Lino et al, 2010;

Nagaishi, Takagi et al, 2011; Zhong, Wang et al, 2012). The proportion of patients with severe bilateral visual disability was even found to be higher in seronegative patients in one study (Nagaishi, Takagi et al, 2011) though the proportion of patients who were blind in at least one eye was no different.

In studies reporting mortality (Adoni, Lino et al, 2010; Akman-Demir, Tüzün et al, 2011; Jarius, Ruprecht et al, 2012), patients who died were all AQP4-Ab positive.

1.7.5 Radiological features

One of the hallmarks of NMO is the longitudinally extensive nature of spinal cord lesions. Several studies have shown no difference in the length of cord lesions between AQP4-Ab positive and negative patients (Adoni, Lino et al, 2010; Jarius, Ruprecht et al, 2012; Zhong, Wang et al, 2012; Kiyat-Atamer, Ekizoglu et al, 2013) although in three of these studies there was a non-significant trend towards longer cord lesions in seropositive patients (Jarius, Ruprecht et al, 2012; Zhong, Wang et al, 2012; Kiyat-Atamer, Ekizoglu et al, 2013). One study (Jarius, Ruprecht et al, 2012) also suggested that very long lesions (>6 vertebral segments) were more common in seropositive patients and that involvement of the entire spinal cord occurred only in seropositive patients. Additionally, this study noted that total lesion load (i.e. adding all cord lesions together) was significantly greater in seropositive patients (Jarius, Ruprecht et al, 2012) and another study showed that whilst there was no difference in length of cord lesions during acute relapses, during remission significantly longer cord lesions were found in seropositive patients (Cabrera-Gomez, Kurtzke et al, 2009). Central grey matter or holocord lesions (Cabrera-Gomez, Kurtzke et al, 2009; Zhong, Wang et al, 2012) have been shown to be more common in seropositive patients, whilst seronegative patients have more peripheral lesions (Zhong, Wang et al, 2012). No differences have been found in the presence of cord atrophy or cavitation between the two groups (Cabrera-Gomez, Kurtzke et al, 2009) nor the proportion of patients with post-contrast enhancement (Zhong, Wang et al, 2012; Kiyat-Atamer, Ekizoglu et al, 2013) or the frequency of cervical or thoracic cord involvement (Zhong, Wang et al, 2012). One study (Kiyat-Atamer,

Ekizoglu et al, 2013) found that cord oedema was significantly more common in seropositive patients but this was not the case in another (Zhong, Wang et al, 2012).

Classic NMO brain lesions i.e. lesions of the hypothalamus and around the 3rd and 4th ventricles corresponding to areas of high AQP4 expression (Nakashima, Fujihara et al, 2006; Pittock, Lennon et al, 2006; Pittock, Weinshenker et al, 2006; Wang, Liu et al, 2011) have largely been reported in studies that either pooled seropositive and seronegative patients in their analyses or only studied seropositive patients (Pittock, Lennon et al, 2006; Pittock, Weinshenker et al, 2006). However, one study described these abnormalities in four NMO patients, two of whom were seronegative (Wang, Liu et al, 2011), though it should be noted that this study used the relatively insensitive tissue IIF assay. A higher proportion of AQP4-Ab positive patients had deep white matter lesions compared to seronegative patients in one study (Cabrera Gomez, Kurtzke et al, 2009) though others found no difference in the frequency of supratentorial or infratentorial brain lesions (Jarius, Ruprecht et al, 2012) nor in the proportion of patients with any brain lesions (Nagaishi, Takagi et al, 2011). A recent study found that the proportion of patients with brain lesions at disease onset was actually higher in seronegative patients (Kiyat-Atamer, Ekizoglu et al, 2013). This study also noted that brain abnormalities in seronegative patients tended to resolve over time, and at follow-up no new lesions appeared, whereas clinically silent new lesions did occur in AQP4-Ab positive patients, though these observations did not reach statistical significance (Kiyat-Atamer, Ekizoglu et al, 2013).

1.7.6 CSF characteristics and biomarkers

CSF characteristics including the frequency of oligoclonal bands (OCBs) and the CSF white cell count have been shown to be similar between seropositive and seronegative patients (Akman-Demir, Tüzün et al, 2011; Nagaishi, Takagi et al, 2011; Jarius, Ruprecht et al, 2012; Kiyat-Atamer, Ekizoglu et al, 2013), with the exception of one study which showed a lower proportion of OCBs in seronegative patients (Bizzoco, Lolli et al, 2009).

There have been many studies reporting the potential of various CSF biomarkers in differentiating NMO from MS but most of these included only seropositive patients or pooled seropositive and seronegative patients. Exceptions include findings of similar CSF MBP (Nagaishi, Takagi et al, 2011) and B-cell activating factor (BAFF) levels (Vaknin-Dembinsky, Brill et al, 2010) and raised CSF GFAP (Petzold, Marignier et al, 2011) and neurofilament heavy chain levels (Miyazawa, Nakashima et al, 2007) in seronegative patients as well as seropositive patients. It was shown recently that CSF proteomic profiles from most seronegative NMO patients were similar to profiles of AQP4-Ab positive patients (Komori, Matsuyama et al, 2012), though some exhibited differences, suggesting that seronegative NMO may be a heterogeneous group of conditions encompassing some patients with disease indistinguishable from seropositive NMO and some patients with a different disease pathogenesis. AQP4-Ab seropositivity was determined with a CBA in this study.

It is also worth noting that although AQP4-Ab levels are markedly higher in serum compared to CSF (Takahashi, Fujihara et al, 2007; Jarius, Franciotta et al, 2010), there have been reports of CSF AQP4-Ab positivity in the absence of serum seropositivity (Klawiter, Alvarez et al, 2009; McKeon, Pittock et al, 2011; Long, Lu et al, 2012; Zhong, Wang et al, 2012; Long, Qiu et al, 2013) although this may be assay-related since three of these five studies used tissue IIF to define AQP4-Ab status.

1.7.7 Treatment response

Immunosuppression is the mainstay of treatment in NMO, and is thought to be effective in reducing relapse rates and stabilising disability. Only a few studies have specifically commented on treatment response in seronegative NMO patients. Relapse rates and EDSS scores before and after treatment with rituximab were shown to be comparable between seropositive and seronegative patients (Bedi, Brown et al, 2011). The ratio of ARR in periods where patients were on oral steroids compared to periods where they were not on oral steroids was shown in one small study to not differ between seronegative and seropositive patients (Watanabe, Misu et al, 2007). Lymphocytapheresis has led to reported improvement in monophasic seronegative NMO

suggesting that this may also be autoantibody-mediated (Nozaki, Hamaguchi et al, 2006; Moreh, Gartsman et al, 2008). However, studies looking at treatment responses in seronegative patients, in common with studies looking at other features of seronegative NMO, have not used CBAs to define serostatus and thus it is difficult to draw definitive conclusions.

As mentioned previously, immunomodulatory treatments commonly used in MS may lead to worsening of NMO. Such reports have been restricted to seropositive patients (Shimizu, Yokoyama et al, 2008; Palace, Leite et al, 2010; Shimizu, Hatanaka et al, 2010; Qian, Cross et al, 2011; Barnett, Prineas et al, 2012; Min, Kim et al 2012) or did not report AQP4-Ab status (Warabi, Matsumoto et al, 2007).

1.7.8 Histopathological features

To date, no studies have reported pathological findings in seronegative NMO patients and thus it is unknown whether these patients share similar features to AQP4-Ab positive patients.

1.7.9 Experimental studies using seronegative patient sera

As discussed, there is now substantial evidence to suggest that AQP4-Ab positive NMO is an astrocytopathy, with patient IgG binding to AQP4 on astrocytes resulting in AQP4 downregulation and astrocyte damage/death, leading to secondary demyelination. Little work has been done to establish whether seronegative NMO sera can also cause astrocyte damage. One study showed that seronegative patient sera could also cause astrocyte death *in vitro* (Sabater, Giralt et al, 2009) whereas another showed that whilst seropositive sera led to astrocyte and oligodendrocyte death *in vitro*, sera from seronegative patients did not (Marignier, Nicolle et al, 2010). Of note, these studies both defined seropositivity by tissue IIF.

In animal models, injection of rats with EAE with human IgG from AQP4-Ab positive but not from seronegative patients results in NMO-like lesions (Bradl, Misu et al, 2009). This may be because seronegative NMO is not antibody-mediated, or because these patients have very low levels of AQP4-Ab, not sufficient enough to cause lesions in rodents.

1.7.10 Presence of other known auto-antibodies in seronegative NMO

It is possible that there is more than one antigen targeted in NMO, as is seen in other autoimmune disorders of the nervous system, such as MG and autoimmune encephalitis. However, despite much interest in seronegative NMO patients, there has been very little published in the literature about possible alternative antigenic targets.

One study (Lalive, Menge et al, 2006a) showed using microarray that purified IgG from one of five seronegative (by tissue IIF) NMO patients contained autoantibodies against Cleavage and Polyadenylation Specificity Factor (a protein required for mature mRNA), ring finger protein 141 and myosin light chain 2, precursor lymphocyte-specific. The role of the latter two proteins in humans is not known and no further studies have reported similar findings.

There have been several case reports of an NMO/NMOSD phenotype occurring in the context of *N*-methyl-D-aspartate receptor (NMDAR) encephalitis (Ishikawa, Tajima et al, 2007; Kruer, Koch et al, 2010; Pennington, Livingstone et al, 2012) and NMO can also occur in association with the paraneoplastic antibody collapsin response mediator protein 5 (CRMP5) (Jarius, Wandinger et al, 2012).

1.7.11 Novel candidate antigens in seronegative NMO

As discussed, NMO with AQP4-Ab appears to be an astrocytopathy. Thus, antibodies to other astrocytic antigens, such as those described in section 1.5.2, may be present in seronegative NMO. Demyelination also occurs in NMO, probably secondary to astrocyte loss in AQP4-Ab mediated disease, but demyelination directly caused by other antibodies could presumably produce an NMO-like phenotype if occurring in the optic nerves and spinal cord. Thus, myelin antigens may be targets in seronegative NMO. One possible target myelin antigen is MOG, which is a CNS-specific antigen that is expressed on the extracellular surface of myelin sheaths (Brunner, Lassman et al, 1989). Although it comprises less than 1% of total myelin protein, its location makes it readily accessible to autoantibodies in the extracellular space and MOG-Ab have recently been identified in paediatric demyelinating disorders, particularly ADEM (O'Connor, McLaughlin et al,

2007; Brilot, Dale et al; 2009; Selter, Brilot et al, 2010; Di Pauli, Mader et al, 2011; Lalive, Hausler, et al 2011). At the time of starting this work, only one study had looked for MOG-Ab specifically in NMO (n=13), none of whom tested positive (McLaughlin, Chitnis et al, 2009).

In animal models, immunisation with MOG peptides leads to EAE (Adelmann, Wood et al, 1995; Johns, Kerlero de Rosbo et al, 1995; Devaux, Enderlin et al, 1997; Storch, Stefferl et al, 1998; Sakuma, Kohyama et al, 2004; Collongues, Chanson et al, 2012) and injection of rodents with T cell mediated EAE with monoclonal antibodies against MOG augments the severity and duration of EAE (Linington, Bradl et al, 1988). MOG-Ab can be detected in the sera of these animals; titres do not correlate with EAE severity and are no different during relapse and remission (Sakuma, Kohyama et al, 2004; Collongues, Chanson et al, 2012).

An NMO-like EAE with selective involvement of the optic nerves and spinal cord develops spontaneously in double-transgenic mice in whom MOG specific T and B cells are present through crossing MOG-specific T cell receptor transgenic mice with MOG-specific Ig-heavy chain knock-in mice (Bettelli, Baeten et al, 2006; Krishnamoorthy, Lassmann et al, 2006). Thus, MOG warrants investigation as a target antigen in NMO.

1.7.12 Techniques for identifying novel antigens in seronegative NMO

In recent years, many novel autoantibodies have been discovered in the sera of patients with neurological diseases. These have been discovered using various techniques, and in general there are two approaches to identifying novel target antigens. The first is a candidate antigen approach, in which known antigens that have potential to be targets in the disease being studied are employed in assays in which patient sera is screened for binding to these specific antigens. These assays include those described in Table 1.3. For example, a candidate antigen approach in seronegative NMO could involve screening patient sera for MOG-Ab using CBAs or ELISA. In the alternative non-candidate antigen approach, used where the antigen is not known or cannot be guessed, various techniques using large numbers of antigens can be used to identify binding to antigens

which can be identified at a later date (Table 1.5). In this thesis, a combination of candidate and non-candidate approaches will be used to identify novel target antigens in seronegative NMO.

1.8 Summary of seronegative NMO

Even using gold-standard AQP4-Ab assays, a proportion of patients who have clinical features of NMO are seronegative for AQP4-Ab. It is currently unclear whether seronegative NMO patients share similar aetio-pathological mechanisms and behave similarly to those with AQP4-Ab in terms of disease presentation, clinical course and disability outcomes. Many studies looking at potential differences between seropositive and seronegative patients have largely been confounded by the use of older, insensitive AQP4-Ab assays, which will result in inclusion of false negative patients, and may have diluted any differences that are present. Additionally, groups often haven't clearly defined what they include in seronegative NMO. Unsurprisingly, therefore, results of these studies are conflicting, with some suggesting that seronegative patients share similar clinical and paraclinical features to AQP4-Ab positive patients, and others finding significant differences.

The clinical and immunological characteristics of seronegative NMO patients defined using sensitive CBAs require further evaluation to determine whether these patients should be grouped together with AQP4-Ab positive patients, or should be considered separately.

Table 1.5. Non-candidate antigen approaches to identify novel target antigens in autoimmune diseases

Technique	General Principles
Western blotting of CNS homogenate	CNS tissue from rodents is homogenised and electrophoresed and probed with patient sera to detect binding to CNS proteins
Culture of CNS cells or cell-lines derived from CNS cells	Primary cultures of CNS cells established from rodents, or cell-lines derived from CNS tissue are incubated with patient sera to detect binding to different cell types
Immunohistochemistry using CNS tissue cryosections	Brains or spinal cord from animals are frozen and a cryostat used to cut thin sections, which are then incubated with patient sera to detect binding to CNS structures
Immunoprecipitation, Western blot and mass spectrometry	Cell-lines or cells from primary culture are incubated with patient sera and bound antibody-antigen complexes isolated through cell solubilisation, captured with protein-G sepharose beads, eluted by heating and then electrophoresed and blotted to visualise precipitated antigens. These proteins can then be identified by mass spectrometry
Phage display library	A cDNA expression library is constructed from brain tissue. Patient IgG is then used to probe the phage library for clones expressing polypeptides recognised by the patient IgG. DNA sequencing of the clones which bind patient IgG can then be performed
Protein microarrays	Large numbers of proteins are attached to a chip, which is incubated with patient sera. Binding is detected with a fluorescent probe

CNS = central nervous system.

1.9 Aims

The main aim of this thesis is to clinically and immunologically characterise patients with NMO and NMOSD.

Specific aims are:

- To characterise the clinical features, disease course and prognostic markers of AQP4-Ab positive NMO/NMOSD
- To investigate whether there may be immunological differences across patients that explain clinical heterogeneity
- To establish whether seronegative NMO/NMOSD patients share similar clinical characteristics with those with AQP4-Ab
- To investigate whether there may be alternative antigenic targets in seronegative NMO/NMOSD

Chapter 2: Materials and Methods

2.1 Patients

The John Radcliffe Hospital in Oxford, together with The Walton Centre for Neurology and Neurosurgery in Liverpool, run a nationally commissioned NHS specialised service for NMO and NMOSD. Patients seen as inpatients at the John Radcliffe Hospital, or as outpatients in the Oxford NMO clinic, were studied in this thesis. Patients with NMO (both ON and TM) and NMOSD, which encompasses patients with simultaneous bilateral ON, recurrent ON, optico-spinal MS and monophasic or recurrent LETM were included.

2.1.1 Clinical database

The Oxford clinical NMO service runs a prospective computerised clinical database, which also contains retrospective data, for AQP4-Ab positive and negative NMO/NMOSD patients.
Collection and storage of anonymised clinical data was approved by the Regional Ethics Committee, reference 10/H0606/56, and informed written consent was provided by the patients.
The following clinical information is collected in the database:

- Demographic information: age, sex, ethnicity
- Clinical features at presentation: anatomical site affected, symptoms and clinical signs, severity of onset attack, treatment, degree of recovery
- Disease course: phenotype, number and severity of relapses, treatment
- Disability outcomes: unilateral visual disability (permanent visual loss in one eye with visual acuity of less than 6/36); bilateral visual disability (permanent bilateral visual loss with visual acuity of less than 6/36 in the best eye), motor disability (permanent inability to walk further than 100 metres unaided), wheelchair dependence and mortality
- Presence of associated autoimmune disorders and other comorbidities

- Radiological features: brain and spinal MRI features at presentation and during the disease course, including the anatomical location and extent of lesions and associated features such as degree of contrast enhancement and spinal cord swelling
- CSF characteristics: cell count, protein and glucose levels and presence or absence of OCBs

2.2 Serum samples

An NMO research tissue bank (Regional Ethics Committee, reference 10/H0606/56) allows collection of serum samples from AQP4-Ab positive and negative NMO/NMOSD patients seen within the Oxford clinical service, together with samples from healthy and autoimmune disease controls (MS and MG). Serum samples are collected at each clinic visit and during relapses, coded and stored in aliquots at -80ºC until required. Some AQP4-Ab negative NMO/NMOSD samples were also provided by The Walton Centre for Neurology and Neurosurgery, Liverpool. This collaboration was approved by their local ethics committee and patients have given written consent.

The cohort for laboratory work comprised 96 samples from 63 patients with NMO/NMOSD testing negative for AQP4-Ab on the Oxford routine diagnostic CBA, 45 AQP4-Ab positive Oxford NMO/NMOSD patients and 56 controls (11 healthy controls, 18 MG controls, 27 MS controls). Further details on the samples used are provided in each chapter of results.

2.3 Cell culture

2.3.1 Maintenance of cell-lines

Cell culture work was performed in a class II laminar flow hood using strict aseptic techniques. Cells were maintained in pre-sterilised plastic tissue culture flasks (75 or 175 cm^2; Corning Incorporated, New York, USA) at 37ºC in a humidified incubator with 5% CO_2.

Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's growth medium (DMEM, Sigma, Ayrshire, UK) supplemented with 10% fetal calf serum (FCS,

PAA laboratories, Pasching, Austria) and 1% penicillin/streptomycin/ampicillin antibiotic solution (PSA; Sigma). Stable cell-lines (HEK293 cells stably transfected with AQP4 isoforms) were maintained in DMEM supplemented with 10% FCS and 100 mg geneticin.

Once 80-90% confluence was reached, cells were detached from flasks with 0.025% trypsin-ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS), resuspended in growth medium and a proportion of the cell resuspension (typically 1/12 for HEK293 cells and 1/10 for stably transfected cell-lines) transferred to a new flask for further growth.

2.3.2 Establishment and maintenance of primary neuroglial cell cultures

Neonatal (P0 to P4) rats were sacrificed and decapitated and the cranium transferred immediately to a dry petri dish on ice. A midline sagittal incision was made in the scalp and skull, which were then retracted. The brain was transferred to a sterile petri dish containing Hanks' Balanced Salt Solution buffer (HBSS; Invitrogen, Paisley, UK) and bisected. The meninges and blood vessels were removed, and the cerebellum and brainstem detached before transferring the forebrain to ice-cold trypsin/HBSS (1:5) solution and incubating at 37^{o}C for 30 minutes. The supernatant was then aspirated and the brain material resuspended in minimum essential medium (MEM 1x alpha medium; Invitrogen) and triturated. The brain/MEM solution was centrifuged for four minutes at 1400 rpm, supernatant aspirated and the trituration process repeated. Further MEM was added and 1ml MEM/cortex solution was aliquoted into each well of a 24-well plate containing glass coverslips that had been freshly treated with Poly-L-Lysine hydrobromide (PLL; Sigma) to promote cell adherence. A ratio of one brain per two 24-well plates was used for neuronal cultures and one brain per four plates for astrocyte cultures. Alternatively, for immunoprecipitation experiments, cultures were established in 175 cm^2 flasks at a ratio of one brain per flask. The plates/flasks were incubated at 37^{o}C for two hours, at which time the MEM was aspirated and replaced by 1 ml/well or 30 ml/flask of Neurobasal medium (NB; Invitrogen) supplemented with glutamine, B27 (50x, Invitrogen) and 1% PSA (NB+++). Cultures were incubated at 37^{o}C in a humidified incubator with 5% CO_2 and every two to three days half of the growth media was aspirated and replaced with fresh NB+++ media. Immunofluorescent staining of astrocytes was

performed after five or six days growth; neurons were stained on days seven to nine. Immunoprecipitation experiments were performed once cells were at least 70% confluent, on days 10 to 15.

2.4 DNA amplification and purification for transfection of cells

Agar media was made by adding 5 g Bacto™Tryptone, 2.5 g Bacto™Yeast, 5 g sodium chloride and 10 g Bacto™Agar (BD Biosciences, Oxford, UK) to 1 L distilled water, which was autoclaved to sterilise. Antibiotics (ampicillin 100 µg/ml or kanamycin 50 µg/ml) were added to the agar media, after which the media was decanted into petri dishes (500 ml per 20 petri dishes) and left at room temperature to cool and solidify. Meanwhile, antibiotic-resistant plasmid DNA at 0.5 to 1.5 µg/µl was diluted 1:10 in nuclease free water and 1 µl of this was used for transformation of 50 µl competent *E. coli* cells thawed on ice from -80ºC in a 1.5 ml eppendorf for 30 minutes. Heat shock was then performed in a water bath at 42ºC for 50 seconds to disrupt the *E. Coli* cell membrane and allow entry of the plasmid. The transformed cells were immediately returned to ice for 3 to 5 minutes. The plasmid/*E. coli* mixture was mixed with 950 µl sterile Luria-Bertani medium (LB; 5 g Bacto™Tryptone, 2.5 g Bacto™Yeast, 5 g sodium chloride), incubated for one hour at 37ºC, centrifuged at 1300 rpm for 5 minutes and the pellet resuspended in 200 µl LB media. The *E. Coli*/plasmid/LB media was spread onto an agar plate containing the antibiotic to which the plasmid encoded resistance (kanamycin for M23R and MOG; ampicillin for M23U). After overnight incubation at 37ºC, a single colony was selected from the agar plate and used to inoculate 10 ml LB media containing 10 µl antibiotic. The inoculated LB media was incubated at 37ºC for 6 to 8 hours, and then transferred to a 3 L glass flask containing 600 ml autoclaved LB media and 650 µl antibiotic for larger scale inoculation. After overnight incubation at 37ºC, plasmid DNA was purified from the inoculated media using a PureYield™ Plasmid Maxiprep System (Promega, Southampton, UK) according to the manufacturer's instructions. The concentration of purified DNA was then calculated using a spectrophotometer and DNA was stored at -20ºC until required.

2.5 Immunofluorescence staining of transfected cell-lines and primary cultures

2.5.1 Transfection of cell-lines for expression of cell surface proteins

HEK293 cells grown to 80-90% confluence were trypsinised, centrifuged and resuspended in growth medium and seeded onto PLL-coated glass coverslips in 6-well plates (4 coverslips per well) at a concentration of 0.4 x 10^6 cells/well. Twenty four hours later, cells were transfected with DNA using standard polyethylenemine (PEI; Sigma) transfection. Briefly, 30 µg of DNA was added to distilled water to a total volume of 75 µl. To this was added 25 µl of 20% glucose solution (in distilled water) and 30 µl PEI. The DNA mixture was left at room temperature for 15 minutes before being added to a 175 cm^2 flask containing HEK293 cells. After 16 to 20 hours, the flask media was aspirated and replaced with fresh media.

2.5.2 Cell-based indirect immunofluorescence assay using cell-lines

Cells were immunostained 36 to 48 hours after transfection. Each coverslip was transferred to a single well of a 24-well plate to which had been added 250 µl of DMEM containing 20 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulphonic acid) (HEPES; Sigma) (DMEM/HEPES) supplemented with 1% bovine serum albumin (BSA) solution and patient serum at a concentration of 1:20. Cells were left to incubate with patient sera for 60 minutes at room temperature, after which time cells were washed three times in PBS and fixed in 4% formaldehyde (in DMEM/HEPES) for 10 minutes at room temperature, before being washed thrice in PBS and incubated with Alexa-Fluor 568 goat-anti-human IgG 1:750 (Invitrogen) for 45 minutes in the dark at room temperature. Cells were then washed three times each in DMEM/HEPES and once in PBS and mounted on glass slides (VWR International, Leighton Buzzard, UK) using fluorescent mounting medium (Dako, California, USA) containing 4',6-diamidino-2-phenylindole (DAPI 1:1000; Invitrogen) to visualise cell nuclei. Slides were left to dry overnight in the dark before being analysed on a fluorescence microscope.

2.5.3 Immunofluorescence staining of primary neuroglial cultures

For immunostaining of primary neuroglial cultures, the growth media was aspirated and replaced with 250 µl per well of NB+++ supplemented with 1% BSA, to which was added 1 µl of patient sera (1:100). Plates were incubated for 60 minutes at room temperature for neuronal staining or overnight at 4°C for astrocyte binding experiments, washed three times with NB+++, fixed for 10 minutes with 4% formaldehyde (diluted in NB+++) and washed a further three times in PBS. Human IgG binding was detected with goat anti-human Alexa-Fluor 488 (1:1000 in NB+++/1% BSA; Invitrogen) incubated for 45 minutes at room temperature. Samples were washed three times in PBS and permeabilised with 0.25% Triton® X-100 (Sigma) for 10 minutes. Staining for neurons, astrocytes or oligodendrocytes was performed by 60 minute incubation with antibodies to neuronal [mouse anti- microtubule-associated protein-2 (MAP-2; 1:1000; Invitrogen)], astrocytic (polyclonal rabbit anti-GFAP; 1:1500; Dako) or oligodendrocyte [mouse anti-2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase); 1:1000; Sigma] markers at room temperature, samples washed three times and bound IgG detected using goat anti-mouse Alexa-Fluor 568 (1:1000; Invitrogen) or goat anti-rabbit Alexa-Fluor 568 (1:1000; Invitrogen) for 45 minutes. Samples were washed three times and then mounted on glass slides using fluorescence mounting media containing DAPI and stored in the dark at 4°C.

2.5.4 Immunofluorescence microscopy and analysis

Immunofluorescence staining of cells was analysed blinded for the presence and intensity of cell surface binding of human IgG. A semi-quantitative scoring system was used as described previously (Leite, Jacob et al, 2008). Briefly, samples were scored on a scale of 0 to 4, where 0 = no binding and 4 = very strong binding.

2.6 FACS to detect human IgG binding to transfected cells

2.6.1 FACS of stable cell-lines to determine M1 and M23 titres

HEK293 cells were stably transfected with either the M1 or M23 AQP4 isoform with or without the red fluorescent protein ds-red, using constructs generated by Dr Patrick Waters. After

transfection, cell-lines were twice sorted using FACS to select the best transfected 10% of cells, which were then cultured and used for experiments. Cells transfected with ds-red/AQP4 are hereafter referred to as M23R and M1R; AQP4-transfected cells not co-transfected with ds-red are referred to as M23U.

For FACS experiments, M23R, M1R and M23U cells were grown to 70 to 80% confluence, trypsinised, centrifuged, counted using a haemocytometer and resuspended in FACS buffer (500 ml DMEM mixed with 2.3 g HEPES supplemented with 1% FCS and 1 mM EDTA) at a concentration of 1 x 10^6/ml in a total volume of 10 ml. M23R and M1R cells were each then mixed in a ratio of 75:25 with uncoloured untransfected HEK293 cells, which acted as an inbuilt negative control. Cells were mechanically rotated at 4^{o}C for one to two hours to recover surface antigen expression and then incubated with patient sera (doubling serial dilutions starting at 1:20) for 30 minutes at 4^{o}C on a rotating wheel (1 x 10^4 cells per sample, total sample volume 200 µl). Cells were then centrifuged for six minutes at 3500 rpm at 4^{o}C, supernatant aspirated, washed with 200 µl FACS buffer, centrifuged once more with aspiration of supernatant and bound human IgG detected by incubating for one hour with 100 µl Alexa-Fluor 488 green fluorescent anti-human IgG (1:500; Invitrogen). The cells were then centrifuged for six minutes at 3500 rpm at 4^{o}C, supernatant aspirated and the cells resuspended in 400 µl of PBS containing 5 mM EDTA, placed on ice and analysed immediately by FACS. Since live cells were used for FACS experiments, all patient sera were heat inactivated for 30 minutes at 57^{o}C to destroy complement prior to use to prevent cell death during experiments. Uncoloured and red controls for FACS calibration consisted of 400 µl cell suspension of untransfected HEK293 cells and M23R cells respectively without incubation with patient sera. M23U cells incubated with AQP4-Ab positive patient sera and anti-human Alexa-Fluor 488 were used as a green control.

2.6.2 FACS of HEK293 cells transiently transfected with the M23 AQP4 isoform

HEK293 cells were cultured in 175 cm^2 culture flasks in DMEM supplemented with 10% FCS and 1% PSA. At 30 to 50% confluence, cells were transfected with a plasmid encoding the human M23-AQP4 isoform with (M23R) or without (M23U) the red fluorescent protein, dsred using

standard PEI transfection as already described. After 16 to 20 hours, the flask media was aspirated and replaced with fresh media. After a further 24 hours, cells were trypsinised and resuspended in FACS buffer and FACS experiments performed as described above. For this diagnostic assay, patient sera was diluted to 1:10 and samples classed as positive if they scored greater than three standard deviations (s.d.) above the mean of control samples.

2.6.3 Objective FACS scoring method

A histogram plot of green fluorescence detected by the fluorescein isothiocyanate (FITC) channel (x axis) against red fluorescence detected by the R-phycoerythrin-Texas red (PE-Texas red) channel (y axis) was created on the FACS analysis software (Summit version 4.3, Dako). A second histogram plot of forward scatter against side scatter was created to enable distinction of whole cells from cell fragments. Uncoloured untransfected HEK293 cells were run first to enable creation of a 'gate' around viable whole cells (R1 gate, Figure 2.1A) to ensure that dead cells or cell fragments were not analysed. Next, unbound M23R or M1R cells were run and two gates created to delineate well transfected cells (R2 gate, Figure 2.1B) and untransfected or poorly transfected cells (R3 gate; Figure 2.1B). R2 gated cells have high dsred levels picked up on the PE-Texas red channel, reflecting high transfection rates with the M23 or M1-AQP4/dsred plasmid. The R2 gate typically contained 30 to 40% of total cells. The R3 gate serves as an 'inbuilt' negative control. Following this set-up process, each sample was analysed in turn. Human AQP4-IgG binding to transfected cells, labelled with Alexa-Fluor 488 anti-human IgG detected using the FITC channel, is seen as a shift in the transfected population (i.e. R2 gate) to the right along the x axis (Figure 2.2A). When patient serum does not contain AQP4-Ab, there is no binding of patient IgG to transfected cells, and thus no shift (Figure 2.2B).

The shift of the transfected cells to the right was quantified by measuring the median fluorescence intensity (MFI). A score for each sample was calculated by subtracting the MFI of the negative control population in gate R3, from the MFI of the well-transfected cells in R2.

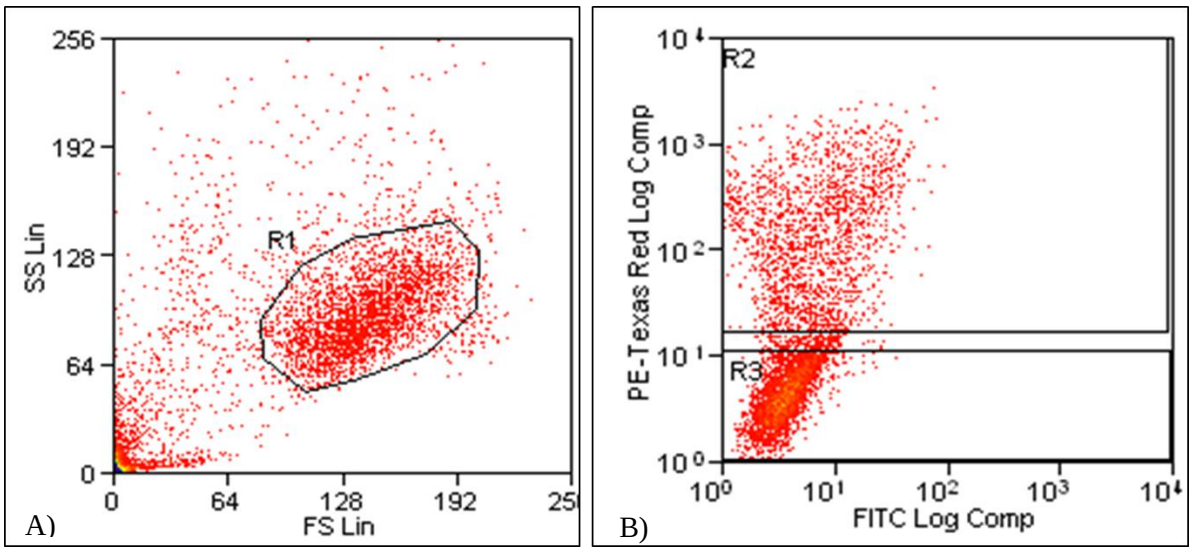


Figure 2.1. Cell gating for FACS experiments. A) Gating of viable uncoloured HEK293 cells (R1 gate) and B) Gating of M23R cells to differentiate well-transfected cells (R2 gate) from poorly-transfected cells (R3 gate). SS = side scatter; FS = forward scatter; Lin = linear scale; Log = logarithmic scale; Comp = compensated; FITC = fluorescein isothiocyanate; PE-Texas Red = R-phycoerythrin-Texas Red.

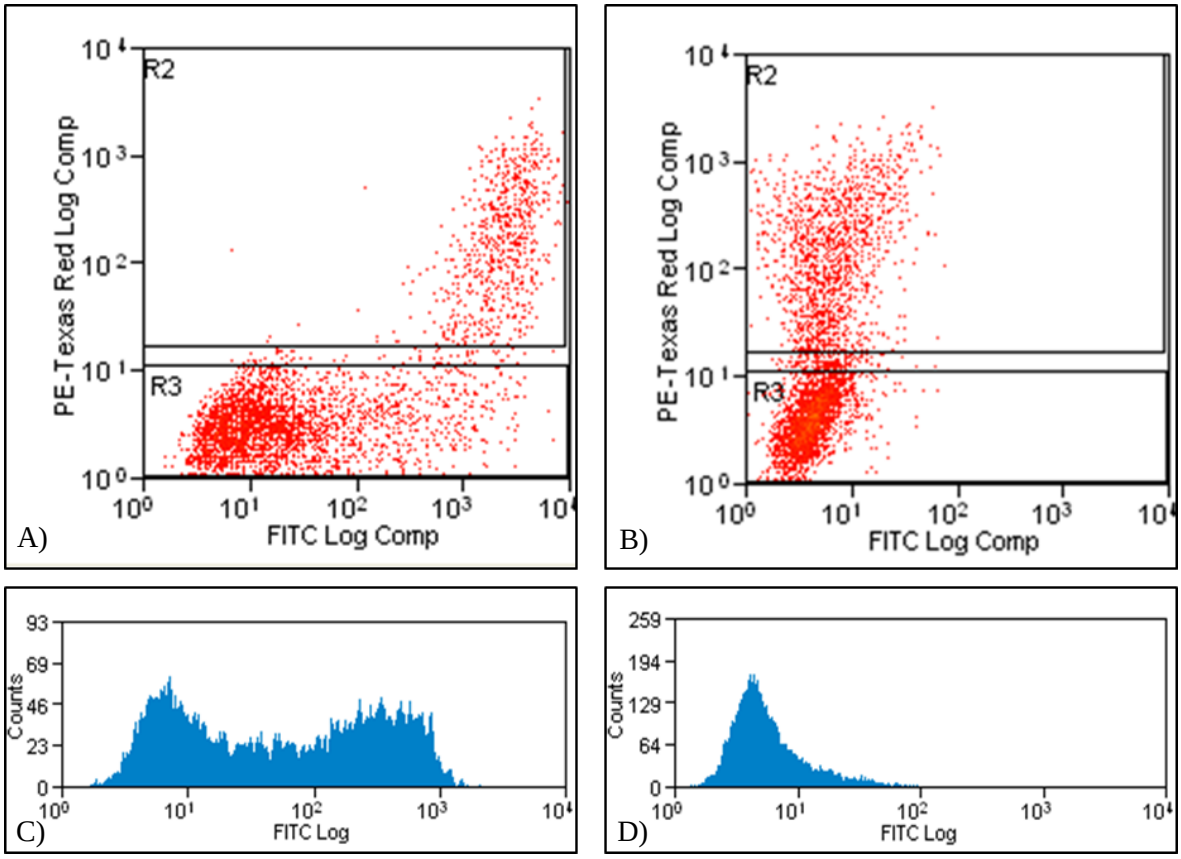


Figure 2.2. Examples of positive and negative results in FACS experiments. Binding of AQP4-Ab in patient sera to AQP4-transfected cells is detected as a shift in the well-transfected cell population (R2 gate) to the right (A), whilst control sera not containing AQP4-Ab does not produce such a shift (B). Although some poorly-transfected cells still show binding of human-IgG, the shift these cells cause in the median fluorescence intensity scores of the negative control R3 gate is minimal. C) and D) show histogram plots demonstrating a cell population that is shifted to the right following incubation with AQP4-Ab positive sera (C) but not control sera (D). The left hand population represents unbound uncoloured HEK293 cells serving as an inbuilt negative control whilst the right hand population represents cells bound by human IgG detected with Alexa Fluor 488 anti-human IgG. Log = logarithmic scale; Comp = compensated; FITC = fluorescein isothiocyanate; PE-Texas Red = R-phycoerythrin-Texas Red.

2.7 Pre-adsorption to remove AQP4-Ab from patient sera

For some FACS and immunoprecipitation experiments, AQP4-Ab were pre-adsorbed from patient sera. Patient sera were diluted to a concentration falling on the linear portion of the AQP4-Ab binding curve for that patient and incubated with AQP4-transfected or untransfected (as a control) HEK293 cells from a single 175 cm^2 flask for 15 minutes at room temperature. Cells were centrifuged and the supernatant harvested and incubated with fresh cells from another 175 cm^2 flask for a further 15 minutes at room temperature. Cells were centrifuged again and the supernatant harvested and used for experiments.

2.8 Electrophoresis and Western blotting

2.8.1 Lithium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting

For electrophoresis experiments, protein solutions were mixed with NuPAGE® lithium dodecyl sulphate polyacrylamide (LDS) sample buffer (Invitrogen) and NuPAGE® reducing agent (Invitrogen) in a ratio of 6.5:2.5:1, heated at 99ºC for 30 minutes and 10-15 µl of the sample was then loaded into a NuPAGE® 4-12% Bis-Tris gradient mini-gel (Invitrogen) and electrophoresed for 30 minutes at 200 V according to the manufacturer's instructions using XCell Surelock® Mini-Cell (Invitrogen) with NuPAGE® 2-(N-morpholino) ethanesulfonic acid (MES) sodium dodecyl sulphate polyacrylamide (SDS) running buffer (Invitrogen). Following this, the gel was stained with EZBlue® (Sigma) to visualise proteins. Alternatively, the gel was transferred to nitrocellulose filter paper (Amersham Hybond-ECL; GE healthcare, Amersham, UK) using an XCell II® Blot module (Invitrogen) in NuPAGE® transfer buffer (Invitrogen) (30 V for one hour). The blot was then washed briefly in PBS and blocked for one hour in PBS/0.005% Triton® X-100/1% FCS/5% skimmed milk powder. Following this, the blot was incubated overnight at 4ºC with polyclonal rabbit anti-AQP4 (Santa Cruz Biotechnology, Santa Cruz, USA; 1:200), washed three times for 10 minutes each in PBS/0.005% Triton® X-100/5% skimmed milk powder (or 5% BSA if biotinylated proteins electrophoresed), incubated with a horseradish peroxidase (HRP)-conjugated secondary

antibody directed against the species of the primary antibody (HRP-conjugated polyclonal goat anti-rabbit 1:1000, Dako) for 45 minutes at room temperature. Alternatively, to visualise biotinylated proteins, the blot was incubated with HRP-conjugated streptavidin (BD Biosciences, used neat in pre-diluted format). Blots were then washed once in PBS and proteins visualised with enhanced chemiluminescence (ECL; GE Healthcare) exposed to photographic film (Amersham Hyperfilm® ECL; GE Healthcare) for one to five minutes or developed by 15 minute incubation with the chromogenic substrate 3,3'-diaminobenzidine (DAB; 0.5 mg in PBS with 0.03% hydrogen peroxide).

2.8.2 LDS-PAGE of cell-lines

Cells grown to confluence in 175 cm^2 flasks were trypsinised, resuspended in growth media, supernatant aspirated and solubilised with 500 µl 1% Triton® buffer [1% Triton® X-100, 150 mM sodium chloride, 0.05 M Trizma® base (Sigma) to pH 8, 0.002 M EDTA, 0.001 M phenylmethylsulfonyl fluoride (PMSF; Sigma) and 10 µl/ml protease inhibitor cocktail (Sigma)] (two 175 cm^2 flasks per sample) for 30 minutes at 4ºC. Cells were centrifuged at 13000 rpm for 15 minutes at 4ºC to remove insoluble nuclei and intracellular organelles and the supernatant transferred to a clean eppendorf. 6.5 µl of supernatant was mixed with 2.5 µl NuPAGE® LDS sample buffer and 1µl NuPAGE® reducing agent, heated at 99ºC for 30 minutes and the sample then electrophoresed as described above.

2.9 Immunocapture and precipitation of cellular membrane proteins

2.9.1 Immunoprecipitation of cell-line cellular membrane proteins by human IgG

Patient serum was diluted 1:20 in 1 ml of DMEM/HEPES/1% FCS/1 mM EDTA and incubated for one hour at 4ºC with cells at >80% confluence detached from one 175 cm^2 flask. After washing three times in PBS, cells were solubilised in 650 µl 1% Triton® buffer for 30 minutes at 4ºC and centrifuged at 13000 rpm for 15 minutes at 4ºC. The supernatant was collected and incubated for two hours at 4ºC with 100 µl protein-G sepharose beads (Sigma) and then centrifuged at 300 rpm for 1 minute to collect a pellet of beads with bound immune complexes. The pellet was then

washed five times in 0.1% Triton® buffer (0.1% Triton® X-100, 150 mM sodium chloride, 0.05 M Trizma base pH 8, 0.002 M EDTA pH 8, 0.001 M PMSF) and proteins eluted by diluting beads in a 1:1 mixture of 0.1% Triton® buffer and NuPAGE® LDS sample buffer with 1% NuPAGE® reducing agent and heating to 99ºC for 30 minutes. After centrifuging for 30 seconds at 300 rpm to separate the supernatant containing eluted proteins from the beads, 10 µl of supernatant was loaded onto a 4-12% Bis-Tris mini-gel and electrophoresed and immunoblotted as described above.

2.9.2 Biotinylation of cell surface membrane proteins

To enable visualisation of unknown antigens in immunoblotting experiments, cells were biotinylated prior to incubation with patient sera. Cells grown to confluence in 175 cm^2 flasks were detached with trypsin, resuspended in growth medium, centrifuged and resuspended in PBS at a concentration of 5 x 10^6 cells/ml in a total volume of 5 ml PBS i.e. 25 x 10^6 cells in total. Cells were washed twice in ice-cold PBS before being incubated for one hour at 4ºC with sulfo-NHS-LC-biotin (0.33 mg in 1 ml PBS; Pierce, Rockford, USA). The biotinylation reaction was quenched by addition of 0.05 M Trizma® base (pH 8) for 5 minutes followed by two washes in 0.05 M Trizma® base and a further wash in ice cold PBS. Immunoprecipitation was then performed as described.

2.10 Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 19 or GraphPad Prism, version 4. Unpaired t-tests or Mann-Whitney U tests were used when comparing two groups. The Kruskal-Wallis test was used when comparing multiple groups. Proportions were compared using Fisher's exact test and chi-square test. The Kaplan-Meier method was used for estimating disability outcomes. Correlations were analysed using Spearman's non-parametric correlation. Cox proportional hazards model was used to compare time-to-event characteristics. Single variable Cox regression was used to assess differences between groups. Statistical significance was set at $p<0.05$.

Chapter 3: Prognostic factors and disease course in AQP4-Ab positive NMO/NMOSD

This is a pre-copyedited author-produced PDF of an article accepted for publication in Brain following peer review. The definitive publisher-authenticated version: Kitley J, Leite MI, Nakashima I, et al., 2012. Prognostic factors and disease course in aquaporin-4 antibody-positive patients with neuromyelitis optica spectrum disorder from the United Kingdom and Japan. Brain 135: 1834-1849 is available online at http://brain.oxfordjournals.org/content/135/6/1834.long

3.1 Introduction

AQP4-Ab are now widely accepted as a biomarker for NMO, and are thought to be pathogenic. It is rare to find AQP4-Ab in healthy individuals or in patients with other neurological conditions (Takahashi, Fujihara et al, 2007; Waters, Jarius et al, 2008; Waters, McKeon et al, 2012) and thus, a positive AQP4-Ab test confirms the diagnosis of NMO with high specificity. This means that AQP4-Ab seropositivity can distinguish NMO from MS and other mimics. Additionally, it has enabled the clinical phenotype of NMO to be broadened to include patients with limited forms of the disease such as monophasic or recurrent LETM or severe, bilateral or recurrent ON or brain/brainstem predominant attacks (Cabrera-Gomez, Bonnan et al, 2009; Bizzoco, Lolli et al, 2009); these limited phenotypes are known as NMO spectrum disorder (NMOSD) (Wingerchuk, Pittock et al, 2007).

It remains to be proven whether patients who have a clinical NMO phenotype but who are AQP4-Ab negative behave similarly to AQP4-Ab positive patients. As mentioned previously, some studies suggest that they may have a different clinical course and outcomes (Nakashima, Fujihara et al, 2006; Kanzaki, Mochizuki et al, 2008; Bizzoco, Lolli et al, 2009; Cabrera-Gomez, Bonnan et al, 2009; Akman-Demir, Tüzün et al, 2011) but previous clinical NMO studies have largely been published prior to the availability of AQP4-Ab testing or included AQP4-Ab seronegative patients in their analyses. Additionally, some excluded patients with clinical or radiological evidence of brain disease, those with monophasic disease or those with limited phenotypes. There have been

no published studies to date characterising the clinical features and reporting disability outcomes and prognostic markers of AQP4-Ab disease.

3.1.1 Aims

The aims of this chapter were to analyse and compare the clinical outcomes and early long-term prognostic characteristics of 106 AQP4-Ab seropositive NMO/NMOSD patients from the United Kingdom (UK) and Japan. Specific aims were:

- To describe the clinical phenotype and disease course of AQP4-Ab positive patients
- To report disability outcomes in AQP4-Ab positive NMO/NMOSD patients
- To identify predictors of disability
- To investigate potential ethnic differences in disease characteristics

3.2 Methods

3.2.1 Patients and clinical data

For this section of work, collaboration was undertaken with The Walton Centre, Liverpool and Tohoku University Hospital, Sendai, Japan. Clinical characteristics, disease course and prognostic characteristics were analysed in 106 consecutively identified AQP4-Ab positive NMO/NMOSD patients followed at one of these three tertiary referral centres. This enabled examination of ethnic differences in AQP4-Ab disease, which have previously been alluded to but not systematically studied. The patients included were identified following the introduction of the AQP4-Ab assay at the centres in 2006. All AQP4-Ab positive patients seen in a specialist NMO clinic or as inpatients at one of the three centres up to September 2010 were included. All patients had tested positive for AQP4-Ab in either the Oxford or the Sendai laboratories using routine diagnostic CBAs (Takahashi, Fujihara et al, 2007; Waters, Jarius et al, 2008).

3.2.2 Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 19. Unpaired t-tests or Mann Whitney U tests were used when comparing two groups. The Kaplan-Meier method was used for

estimating disability outcomes. Cox proportional hazards model was used to compare time-to-event characteristics. Single variable Cox regression was used to assess differences between groups. Statistical significance was set at $p<0.05$.

3.3 Results

3.3.1 Demographic data

One hundred and six AQP4-Ab seropositive NMO/NMOSD patients were included; 59 from the UK (Oxford and Liverpool) and 47 from Sendai, Japan. The cohort had a median disease duration at last follow-up of 75 months (range 3 to 417 months). Disease duration was significantly longer in the Japanese cohort than in the UK cohort (median 115 vs. 60 months; p=0.041). The UK cohort was mainly Caucasian (76%). The remainder were Afro-Caribbean (20%) and Asian (3%). All Japanese patients were Asian. The mean age at disease onset was 40.5 ± 16.1 years (range 2.7 to 76.7 years) and this was similar in the two cohorts. The majority of patients (87%) were female – 81% of the UK cohort and 98% of the Japanese cohort. The few male patients had comparable general characteristics to the females with similar mean age at disease onset (39.4 ± 20.9 years vs. 40.6 ± 15.5 years; p=0.801) and similar disease duration (median 53 vs. 75 months; p=0.901).

3.3.2 Disease Presentation and Course

The majority of patients (84%) presented with either ON or LETM; only 4% presented with a classic Devic's syndrome of simultaneous ON and LETM (Table 3.1).

The mean age at disease onset of patients presenting with ON was significantly younger than patients presenting with LETM (37.1 ± 16.6 years vs. 45.7 ± 15.0 years; p=0.01) and this was the case in both cohorts. Of patients with disease onset <30 years of age, 61% presented with ON compared to only 18% with LETM, whereas of patients with disease onset >50 years of age, 66% presented with LETM compared to 28% presenting with ON (Table 3.2).

Eighty six per cent of the patients experienced a relapsing course (similar in both cohorts) and no patients developed a progressive stage. Overall, 49% experienced a relapse within one year of

Table 3.1. Comparison of demographic and clinical features between UK and Japanese cohorts

	Total	UK			Japanese
		Total	Caucasian	Afro-Caribbean	
No. patients	106	59	45	12	47
Mean age at onset (range)	40.5 (2.7 to 76.7)	40.6 (2.8 to 76.8)	44.9 (7.2 to 76.8)	28.0 (2.8 to 48.5)	40.1 (13 to 73)
% female	87	81	82	75	98
Median disease duration, months (range)	75 (3 to 417)	60 (3 to 417)**	60 (3 to 417)	46 (23 to 174)	115 (7 to 392)**
Onset attack					
LETM	43%	49%	53%	33%	36%
ON	41%	37%	38%	33%	45%
ON & LETM	4%	5%	5%	8%	2%
Brain/Brainstem	5%	2%	2%	0%	9%
Mixed e.g. ON & brain	7%	7%	0%	25%	9%
Disease course					
Relapsing	86%	86%	82%	100%	85%
Monophasic	14%	14%	18%	0%	15%
Phenotype					
NMO*	53%	53%	44%	75%	53%
rLETM	17%	20%	25%	8%	13%
rON	7%	8%	11%	0%	6%
mON	4%	2%	2%	0%	6%
mLETM	9%	12%	16%	0%	6%
mbrain/BS	1%	0%	0%	0%	2%
other e.g. brain & ON	8%	5%	2%	17%	13%
Median time to first relapse (months)	14	12	12	12	15
Mean annualised relapse rate	0.82	0.97†	0.98	0.97	0.63†
% reaching visual endpoint	18	22	16	42	12
% reaching motor disability endpoint	34	46††	49	33	18††
% reaching wheelchair endpoint	23	29	31	25	15
% died	9	14	16	8	4

*at least one attack of myelitis with LETM on spinal cord imaging, and at least one attack of optic neuritis; **p=0.041; †p=0.020; ††p=0.0019. No. = number; BS = brainstem; LETM = longitudinally extensive transverse myelitis; ON = optic neuritis; r = relapsing; m = monophasic.

Table 3.2. Differences in type of onset attack and disability outcomes by age at disease onset

Onset age (years)	N	Median follow-up, months (range)	N/% presenting with ON	N/% of ON-onset reaching visual endpoint	N/% of ON-onset reaching motor endpoint	N/% presenting with LETM	N/% of LETM-onset reaching motor endpoint	N/% of LETM-onset reaching visual endpoint
<30	28	79 (12 to 394)	17 (61%)*	8 (47%)	1 (6%)†	5 (18%)**	1 (20%)	1 (20%)
30 - 50	49	86 (6 to 417)	18 (37%)	4 (22%)	4 (22%)	22 (45%)	9 (41%)	1 (5%)
>50	29	60 (3 to 229)	8 (28%)*	1 (12.5%)	5 (63%)†	19 (66%)**	13 (68%)	1 (5%)

*p=0.0167; †p=0.0055; **p=0.0004. N = number; ON = optic neuritis; LETM = longitudinally extensive transverse myelitis.

disease onset and 70% within two years. If patients started on long-term immunosuppression before their first relapse were excluded, these figures rose to 61% and 81% respectively. Survival analysis revealed that the median time to first relapse was 14 months (range 1 to 179; 95% confidence interval (CI) 10.4 to 17.6) and this was similar in the two cohorts (12 months UK cohort, 15 months Japanese cohort; p=0.461). Of note, four patients had a delay (without immunosuppressant treatment) of greater than 10 years between disease onset and their first relapse. The mean ARR was 0.82 ± 0.78.

Patients with a monophasic illness had a significantly shorter follow-up compared to those with relapsing disease (median 19 vs. 106 months; $p<0.001$) and were treated earlier. They were also significantly older at disease onset than those with relapsing disease (mean 50.4 ± 15.1 vs. 38.9 ± 15.8 years; p=0.01).

The majority of patients with a relapsing course experienced attacks of both ON and LETM (62%; i.e. developed NMO) but 18 patients (20%) had attacks of only LETM and 8 (9%) experienced relapsing ON. Some patients had myelitis with short cord lesions during the course of their disease, but all such patients additionally had attacks of LETM. Forty seven per cent of patients did not develop NMO according to the Wingerchuk 2006 diagnostic criteria. Six patients (three from each cohort) experienced a delay of greater than 10 years between disease onset and development of NMO.

Time to first relapse and time to NMO were not significantly influenced by gender, age at disease onset or onset attack severity. Time to first relapse was not influenced by the type of onset attack (Figure 3.1A) but patients presenting with ON had a significantly greater cumulative probability of progressing to NMO over time than patients presenting with LETM (Figure 3.1B).

There were no marked differences between the UK and Japanese cohorts in the proportion of onset symptoms, the percentage that developed a relapsing course, the time to first relapse, the proportion that developed NMO or the time to developing NMO. However, the mean ARR was

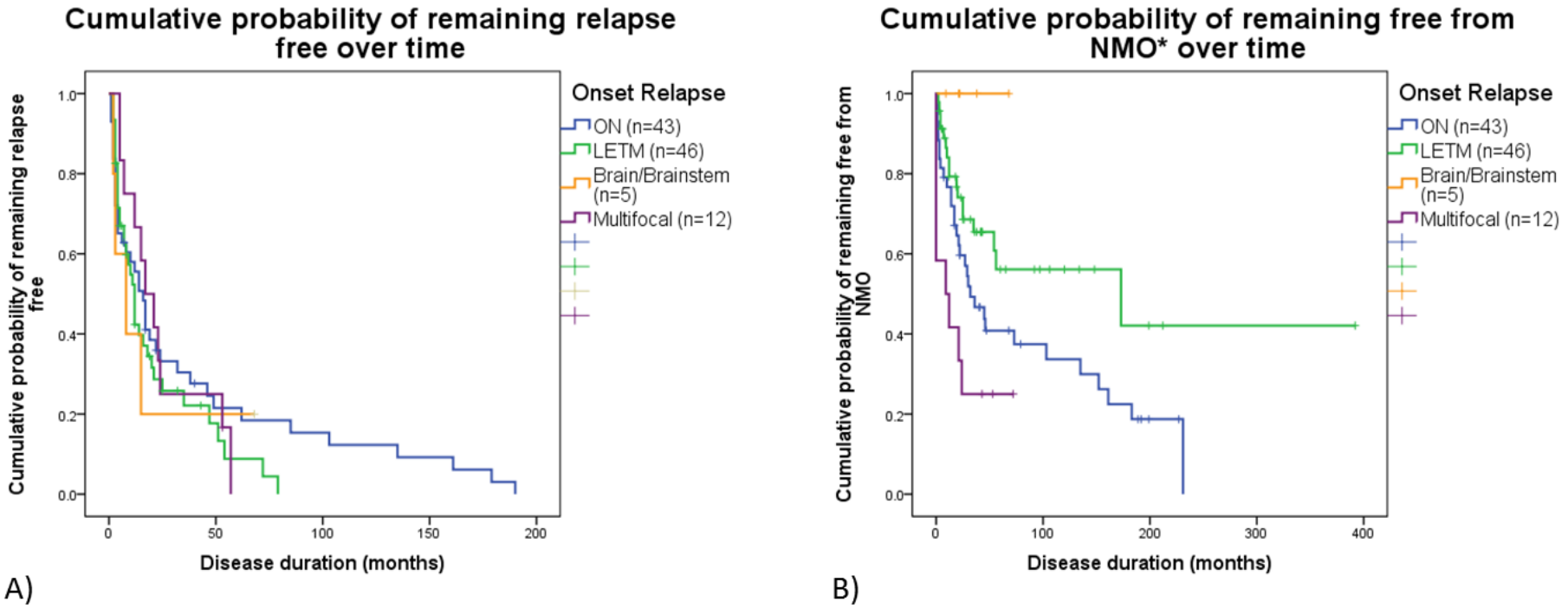


Figure 3.1. Probability of developing relapsing disease and NMO depending on onset relapse type. Kaplan-Meier curves showing cumulative probability over time of remaining free from relapse (A) and free from progression to NMO (B) depending on onset relapse type. A) Time to first relapse was not influenced by type of onset attack. B) Patients presenting with LETM were significantly less likely to develop NMO over time than patients presenting with ON (37% vs. 72%; hazard ratio (HR) 0.539; 95% CI 0.298 to 0.977; p=0.042). No patients presenting with brain disease progressed to NMO. *NMO = at least one attack of ON and at least one attack of LETM.

significantly higher in the UK cohort than in the Japanese cohort (0.97 ± 0.87 vs. 0.63 ± 0.59; p=0.020).

3.3.3 Disability

Morbidity and mortality within the whole group were substantial, with around one fifth (18%) developing permanent visual disability (defined as bilateral visual disability with acuity in best eye of 6/36 or worse), one third (34%) developing permanent motor disability (defined as inability to walk 100 metres unaided), and nearly one quarter (23%) becoming wheelchair dependent by last follow-up after median disease duration of 75 months. Nine per cent of patients died. Table 3.3 summarises predictors of visual disability, motor disability and mortality.

3.3.3.1 Visual Disability

Ethnicity (see later), gender, age at disease onset and type and severity of onset attack appeared to be important predictors of permanent visual disability.

Males were significantly more likely to reach the visual disability endpoint over time than females (Figure 3.2A). As there was only one male in the Japanese cohort, the analysis was also performed on the UK cohort only and the result remained the same. ON-onset patients were significantly more likely than LETM-onset patients to develop visual disability (Figure 3.2B) and patients with severe onset attacks were significantly more likely to develop visual disability over time than those presenting with non-severe attacks (Figure 3.2C); this result remained significant even when stratifying for age.

Older onset patients were less likely to become visually disabled (Figure 3.2D) with a hazard ratio (HR) of 0.62 for each year of increasing age at disease onset and this remained significant even when stratifying for onset symptom type (HR 0.66 for each year; 95% CI 0.46 to 0.93; p=0.017). Patients who reached the visual disability endpoint were significantly younger at disease onset than those who did not (mean 30.2 ± 15.7 years vs. 42.8 ± 15.3 years; p=0.004). Nearly half of those with disease onset <30 years reached the visual disability endpoint (11 of 28 patients) compared to just 2 of 29 old-onset patients (onset >50 years; Table 3.2). This age effect was attributable to the

Table 3.3. Predictors of disability in AQP4-Ab positive NMO/NMOSD

	Visual disability (best eye worse than 6/36)	**Motor disability (unable to walk >100 metres unaided)**	**Wheelchair dependence**	**Death**
Gender	Males more likely than females (p=0.003; HR 4.9)	No difference (p=0.844)	No difference (p=0.551)	No difference (p=0.944)
Ethnicity	Higher probability in Afro-Caribbeans than in Caucasians (p=0.046; HR 1.83) or Asians (p=0.030; HR 3.69)	Caucasians more likely than Asians (p=0.001; HR 3.85)	Caucasians more likely than Asians (p=0.026; HR 2.82)	Asians less likely than Caucasians (p=0.032; HR 0.18)
Onset age	Lower risk with increasing age (p=0.007; HR 0.62 for each year); due to UK cohort effect (no difference in Japanese cohort)	Higher risk with increasing age (p<0.001; HR 1.82 for each year). More profound effect in Japanese cohort	Higher risk with increasing age (p<0.001; HR 1.92 for each year). More profound effect in Japanese cohort	Higher risk with increasing age (p=0.003; HR 2.12 for each year). Effect seen in both cohorts
Onset relapse type	ON more likely than LETM (p=0.023; HR 4.31)	LETM more likely than ON (p=0.004; HR 3.00). Due to effect in UK cohort only	No difference (p=0.188)	No difference (p=0.137)
Onset relapse severity	Severe onset relapse more likely (p=0.003; HR 4.6)	No difference (p=0.132)	No difference (p=0.256)	No difference (p=0.591)

HR = Hazard ratio; ON = optic neuritis; LETM = longitudinally extensive transverse myelitis.

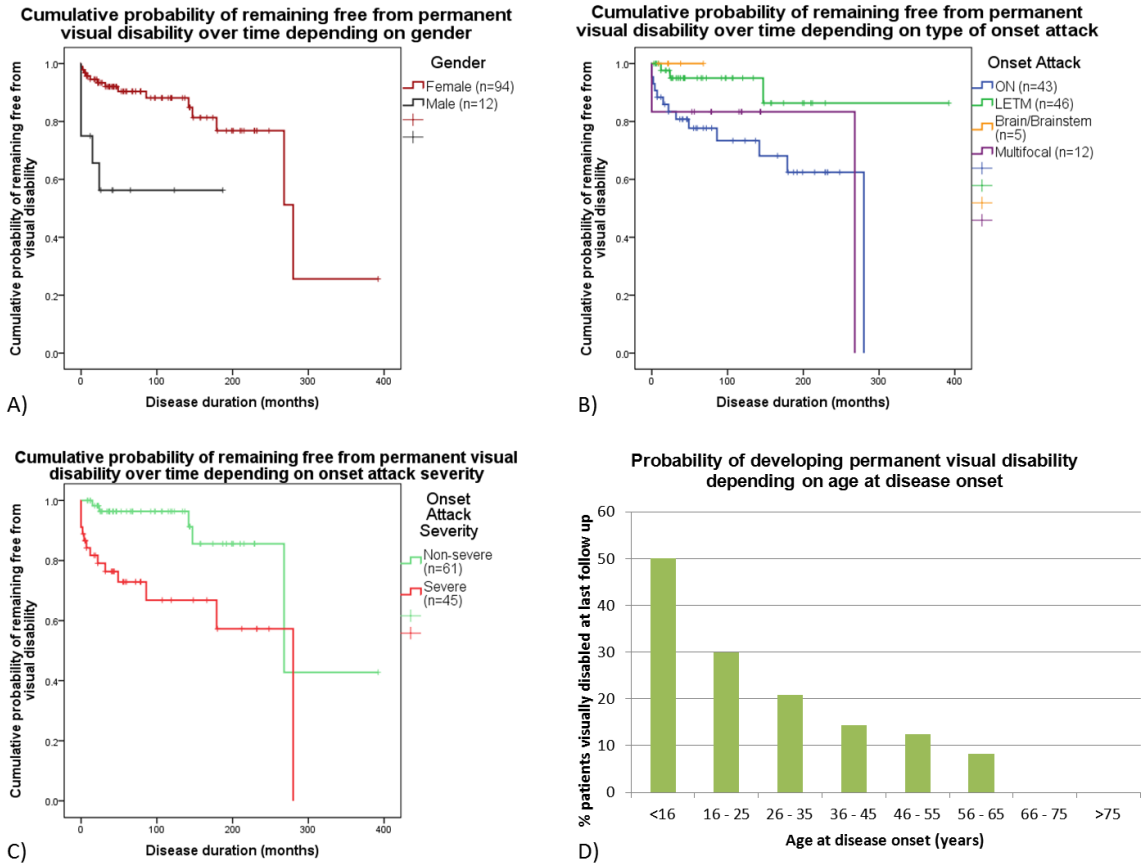


Figure 3.2. Predictors of visual disability in AQP4-Ab positive NMO/NMOSD. A-C) Kaplan-Meier curves showing cumulative probability over time of remaining free from permanent visual disability (defined as visual acuity of worse than 6/36 in the best eye) depending on gender (A), type of onset attack (B) and severity of onset attack (C). A) Males were significantly more likely than females to develop permanent visual disability over time (HR 4.9; 95% CI 1.70 to 14.1; p=0.003); B) patients presenting with ON were significantly more likely to develop visual disability over time than those presenting with LETM (HR 4.31; 95% CI 1.223 to 15.15; p=0.023), though this result became non-significant when stratifying for age at disease onset. No patients presenting with only brain disease developed permanent visual disability over the follow-up period; C) patients with severe onset relapses (visual acuity 6/60 or worse at nadir for ON or unable to walk at nadir for LETM) were significantly more likely to develop visual disability over time than those presenting with non-severe attacks (HR 4.6; 95% CI 1.65 to 12.84; p=0.003) and this result remained significant when stratifying for age at disease onset. D) Bar chart demonstrating that the likelihood of developing visual disability over time was significantly associated with age at disease onset, with older patients less likely to become visually disabled (HR 0.91 for each year of increasing age; 95% CI 0.85 to 0.97; p=0.007). There were no significant differences in follow-up durations between the different age groups to account for these findings (p=0.300).

UK cohort (HR 0.60 for each year; 95% CI 0.41 to 0.89; p=0.007). In the Japanese cohort, visual disability was not related to onset age.

3.3.3.2 Motor Disability

Important predictors of permanent motor disability were ethnicity (see later) and age at disease onset. Motor disability was not related to gender or onset relapse severity.

Older onset age was predictive of reaching 'inability to walk >100 metres unaided' outcome (Figure 3.3A; p<0.001) and becoming wheelchair dependent (Figure 3.3B; p<0.001). This effect was more profound in the Japanese group than in the UK group. Even when stratifying for onset relapse type, older age at disease onset was predictive of motor disability (p<0.001).

Patients with an LETM-onset attack had a significantly higher probability of developing motor disability over time than patients presenting with ON (50% vs. 23%; HR 3.00; 95% CI 1.42 to 6.37; p=0.004). However, as older patients were more likely to present with LETM we stratified for age at disease onset which led to this result becoming non-significant. Even ON-onset patients had a high risk of developing motor disability if age at onset was >50 years whilst LETM-onset patients presenting <30 years had a low risk of developing motor disability (Table 3.2).

3.3.3.3 Mortality

Ten of the 106 patients (9.4%) died after a median disease duration (within this group) of 99 months (range 5 to 229) and a mean number of relapses of 4.5 (range 1 to 10). Death was due to NMO or complications related to NMO in seven of the 10 patients. One patient died from choking on a food bolus as a result of impaired bulbar function, three severely disabled patients died from bronchopneumonia and one from sepsis, one patient died from respiratory failure after requiring home non-invasive ventilation and one patient developed respiratory failure during a severe myelitis. Of the non-NMO-related deaths, one patient died from a myocardial infarction, one from a pulmonary embolism and one from accidental choking without any history of bulbar dysfunction. The mean age at death was 52 ± 16 years (range 17 to 72) and older age at disease onset was

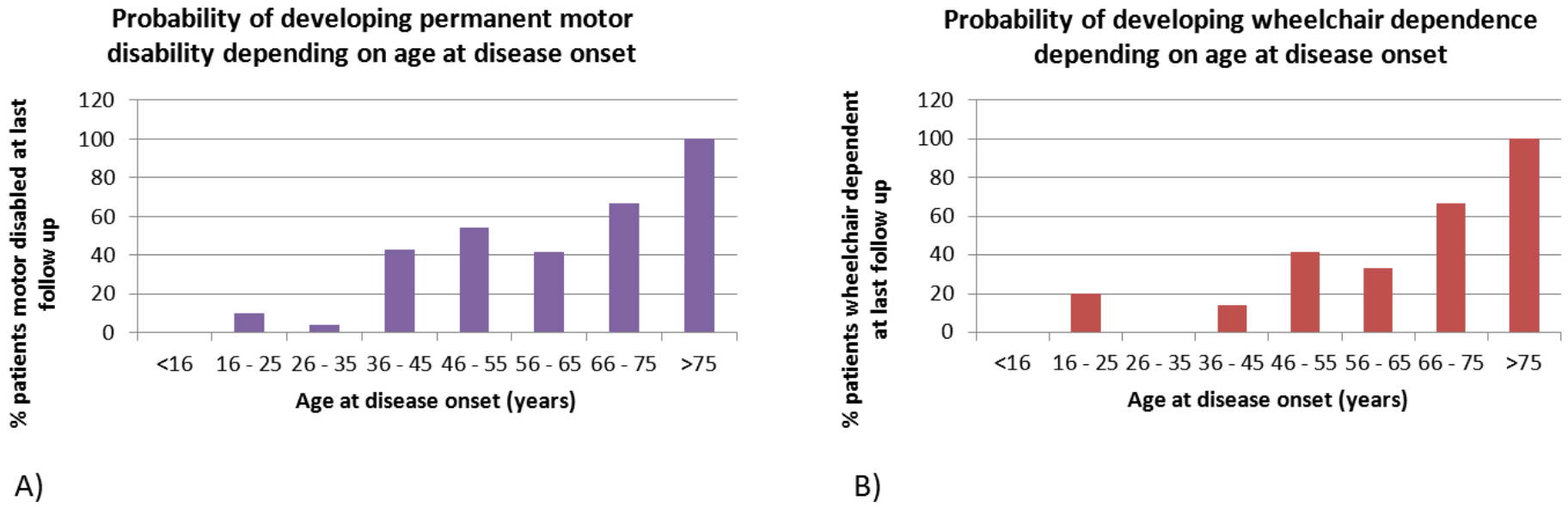


Figure 3.3. Likelihood of developing motor disability depending on age at disease onset. Bar charts showing that the probability of developing permanent motor disability (defined as inability to walk >100 metres unaided) (A) and wheelchair dependence (B) increased with increasing age at disease onset. Older onset age was significantly associated with likelihood of developing permanent motor disability (HR 1.82 for each year of increasing age; 95% CI 1.45 to 2.28; $p<0.001$) and wheelchair dependence (HR 1.92 for each year of increasing age; 95% CI 1.34 to 2.74; $p<0.001$). This effect was more profound in the Japanese group than in the UK group (for walk >100 metres unaided, HR 2.37 for each increasing year; 95% CI 1.48 to 3.81 vs. HR 1.6; 95% CI 1.27 to 2.04; $p<0.001$; for wheelchair dependence, HR 3.33; 95% CI 1.72 to 6.40 vs. HR 1.92; 95% CI 1.34 to 2.74; $p<0.001$). There were no significant differences in follow-up durations between the different age groups to account for these findings ($p=0.300$).

strongly predictive of death (HR 2.12 per year; 95% CI 1.29 to 3.49; p=0.003). Mortality was not associated with gender, onset attack severity nor type of onset attack.

3.3.4 Treatment Effects

Treatment data were incomplete for four patients, and one (Japanese) patient did not receive long term immunosuppression (defined as use of maintenance corticosteroids beyond those used to treat an acute relapse, or treatment with a steroid-sparing agent). Considering the remaining 101 patients, time to treatment with immunosuppression was significantly shorter in the UK than in the Japanese cohort [median 13 (range 0 to 276) vs. 41.5 (range 0 to 272) months; p=0.0131]. Twenty three patients (15 from the UK, 8 Japanese) received immunosuppression before their first relapse. These patients had a significantly longer time to first relapse than those who were not treated prior to their first relapse (median 57 months (95% CI 45.7 to 68.3) vs. 9.0 months (95% CI 5.7 to 12.3); p<0.001). Additionally, patients who were treated before their first relapse were significantly less likely to develop NMO over time than those who were not treated before their first relapse (HR 0.371; 95% CI 0.148 to 0.931; p=0.035).

Of these 23 patients treated early, seven were left motor disabled after their index event, of which four were left wheelchair dependent. Thus the bias towards early treatment for those with severe onset attacks means that early therapy (treatment before the first relapse) appears to be associated with greater motor disability when compared to later treatment (Figure 3.4A). This observation did not carry over to becoming wheelchair dependent or to death. There was a trend towards early treatment being protective against development of visual disability, and in fact no patients who were treated before their first relapse reached the visual disability endpoint (Figure 3.4B). However, due to the small numbers of patients being treated before their second attack, this did not reach statistical significance (p=0.219). It should be noted that a severely disabling index LETM could lead to a motor disability endpoint but a severe onset unilateral ON could not lead to our definition of visual disability (bilateral visual impairment). Thus, this may explain why early treatment appeared to delay visual but not motor outcomes.

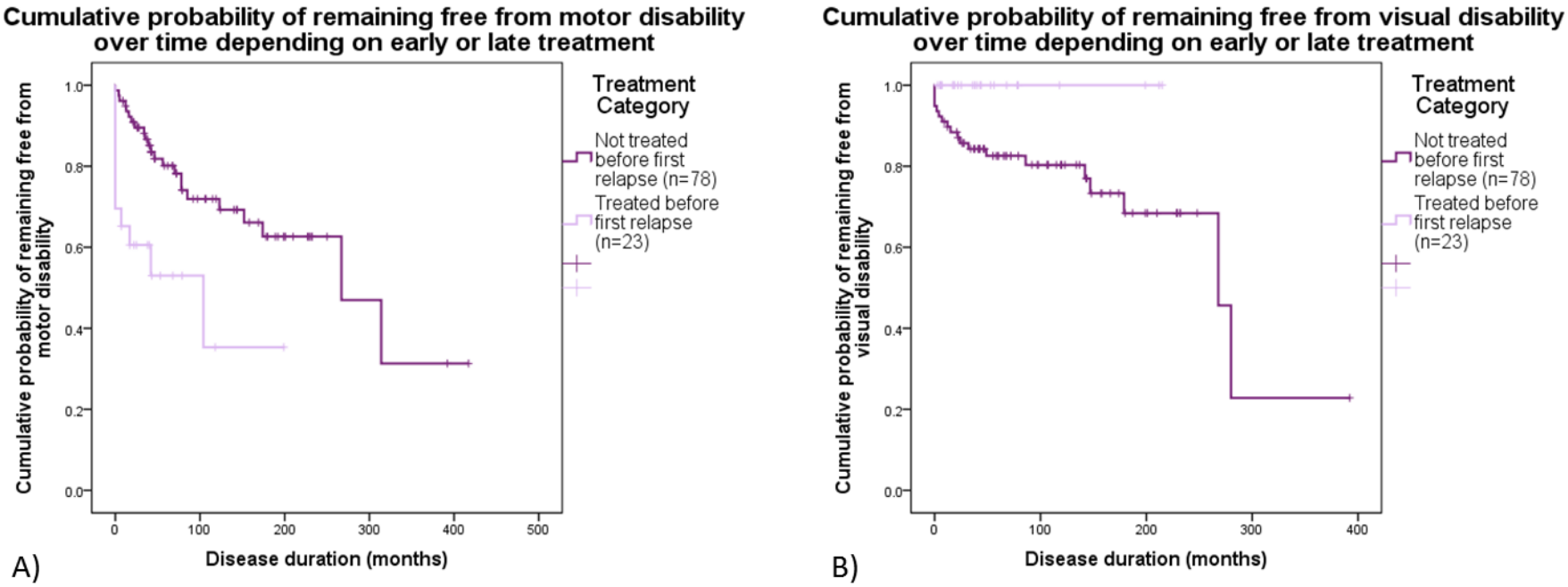


Figure 3.4. Effect of early treatment on disability outcomes. Kaplan-Meier curves to demonstrate effects of early prophylactic immunosuppression on disability endpoints showing A) patients treated before their first relapse were significantly more likely to develop motor disability over time than those whose treatment was delayed (HR 4.40; 95% CI 1.83 to 10.61; p=0.001), probably because many patients presenting with myelitis reached the motor disability endpoint during the index event and were started early on immunosuppression. B) No patients treated with prophylactic immunosuppression before their first relapse reached the visual endpoint, suggesting that early treatment is associated with a reduced likelihood of visual disability.

ARR was calculated before and after treatment with immunosuppression. Mean ARR pre-treatment (excluding the index event) was 1.34 (± 1.22). Mean ARR post-treatment was 0.22 (±0.43), which was a significant reduction ($p<0.01$). Fifty out of the 101 patients for whom treatment data were available had no relapses after commencing treatment (50%), over a median follow-up period on treatment of 33 months (range 1 to 123 months).

Of note, 11 patients (two UK, nine Japanese) were treated with interferon-beta prior to commencing immunosuppressant therapy. Interferon-beta was stopped because of adverse effects in five patients and because of on-going or increasing relapses in six patients.

3.3.5 Outcomes of the UK and Japanese cohorts

Table 3.1 summarises the main characteristics of each cohort. Despite the shorter follow-up time, patients from the UK cohort appeared to have more severe disease and were more likely to reach each disability endpoint than patients from the Japanese cohort (Figure 3.5 and Table 3.1). The residual disability left by the onset attack was worse in the UK cohort. Onset LETM events were severe (unable to walk unaided at nadir) in 11 UK patients (19%) and one Japanese patient (2%). Seven patients, all from the UK, were left with permanent motor disability (unable to walk >100m unaided) after the index event, of whom four were left wheelchair dependent. Although more Japanese patients (n=14; 30%) than UK patients (n=13; 22%) had severe onset ON events (visual acuity in affected eye 6/60 or worse at nadir), the four patients left with permanent bilateral visual disability after this onset attack (visual acuity <6/36 in best eye) were all from the UK (two Caucasian, two Afro-Caribbean). Therefore, overall, none of the Japanese cohort reached any of the disability endpoints as a result of their onset attack, whilst 11 (19%) of the UK patients did.

There was a trend towards more patients in the UK cohort reaching the permanent visual disability endpoint over time (Figure 3.5A) and overall this took a mean of 2.9 ON relapses (2.6 UK; 3.5 Japanese). The Japanese cohort had a significantly lower probability of developing motor disability and wheelchair dependence over time than the UK cohort (Figures 3.5B and 3.5C). The Japanese cohort required a mean of 2.9 LETM relapses compared to 1.9 in the UK cohort to reach the

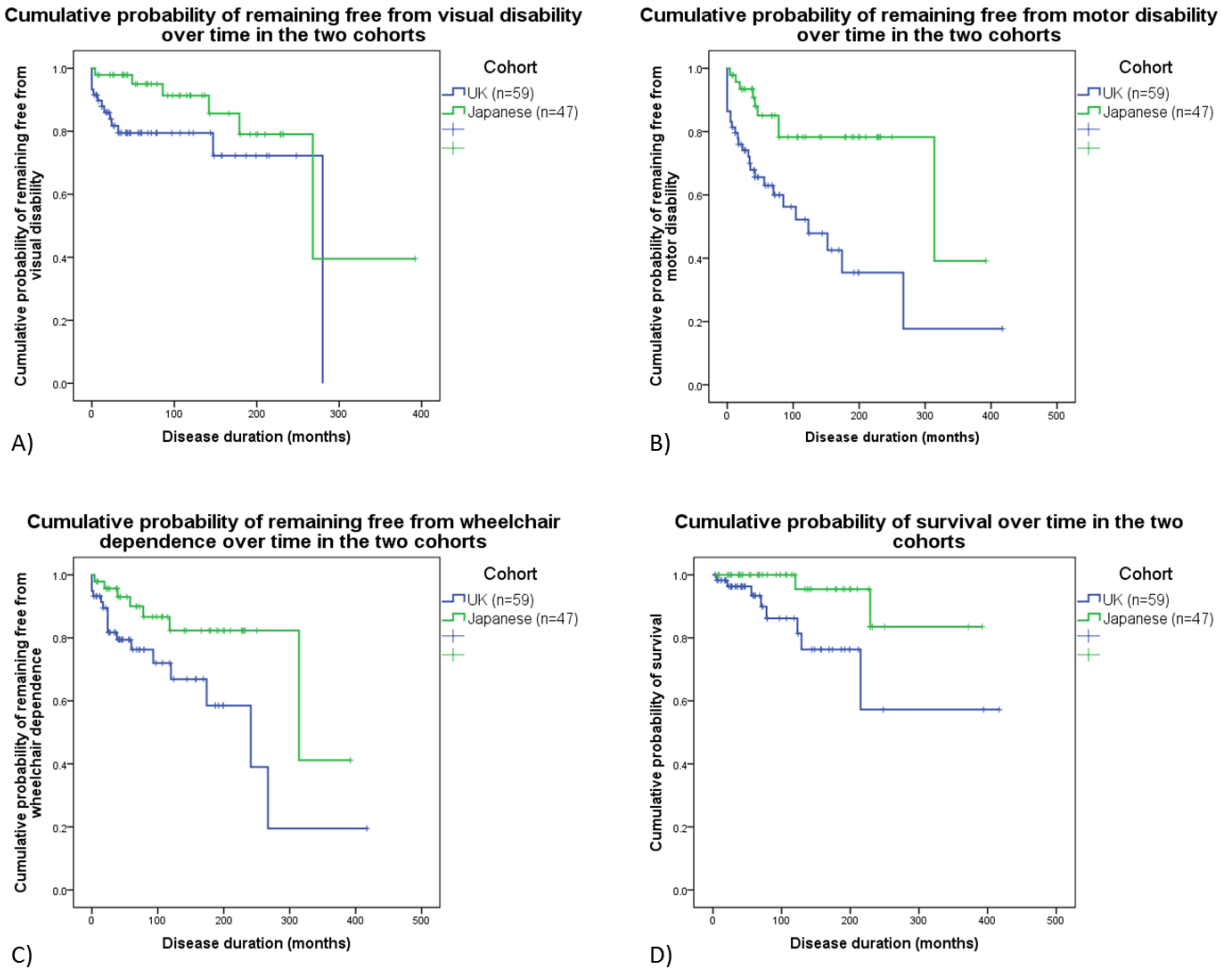


Figure 3.5. Differences in disability outcomes between the UK and Japanese cohorts. Kaplan Meier analysis of time to disability endpoints in the UK and Japanese cohorts showing A) a trend towards greater likelihood of developing visual disability over time in the UK vs. Japanese cohort; 22% of the UK patients reached the visual endpoint (visual acuity worse than 6/36 in best eye) compared to just 12% of the Japanese cohort but this did not reach statistical significance (p=0.08); B) the Japanese cohort were significantly less likely to develop permanent motor disability (unable to walk >100 metres unaided) than the UK cohort (18% vs. 46%; HR 0.305; 95% CI 0.142 to 0.652; p=0.002); C) the Japanese cohort were significantly less likely to become wheelchair dependent compared to the UK cohort (15% vs. 29%; HR 0.388; 95% CI 0.160 to 0.942; p=0.036) and D) the Japanese cohort were significantly less likely to die over the follow-up period than the UK cohort (4% vs. 14%; HR 0.139; 95% CI 0.029 to 0.909; p=0.038).

'unable to walk >100 metres unaided' end point and 3.9 relapses compared to 2.9 in the UK cohort to become wheelchair dependant. The UK cohort had a greater mortality risk with time than the Japanese cohort (Figure 3.5D).

3.3.6 Comparisons between ethnic groups

Because of the differences noted between the UK and Japanese (all Asian) cohorts, the UK cohort was further divided into ethnic groups. Because the 59 UK patients were composed of 45 Caucasians, 12 Afro-Caribbeans and only two Asians, only differences between the Caucasian, Afro-Caribbean and Japanese Asian groups were investigated; the two UK Asians were not included in analyses.

Table 3.1 summarises the differences between ethnic groups. There were similar gender ratios in the three groups but Afro-Caribbean patients had a significantly younger age at disease onset than Caucasian patients (mean 28.0 ± 13.1 vs. 44.9 ± 17.2 years; p=0.003) and Japanese patients (mean 40.1 ± 13.55; p=0.008). Whilst mean age at disease onset did not differ significantly between Caucasians and Japanese Asians, distribution of onset age did (Figure 3.6).

Afro-Caribbean patients commonly had a multifocal index event involving the brain and/or brainstem (commonly including ON), but this was rare in Caucasians and the Japanese cohort (Table 3.1). All Afro-Caribbean patients followed a relapsing disease course compared to around 80% in the other groups. Time to first relapse was similar in the three ethnic groups (Figure 3.7A). The mean ARR was significantly higher in Caucasians and Afro-Caribbeans than in the Japanese patients. Compared to the other groups, more Afro-Caribbean patients developed an NMO phenotype over time (Figure 3.7B).

Afro-Caribbean and Asian patients had a higher proportion of relapses involving the brain than Caucasian patients, whilst spinal cord relapses were more common in Caucasian patients than in other ethnic groups (Figure 3.8). Afro-Caribbean patients had a greater probability of developing permanent visual disability (Figure 3.9A) whilst those of Caucasian ethnicity were significantly

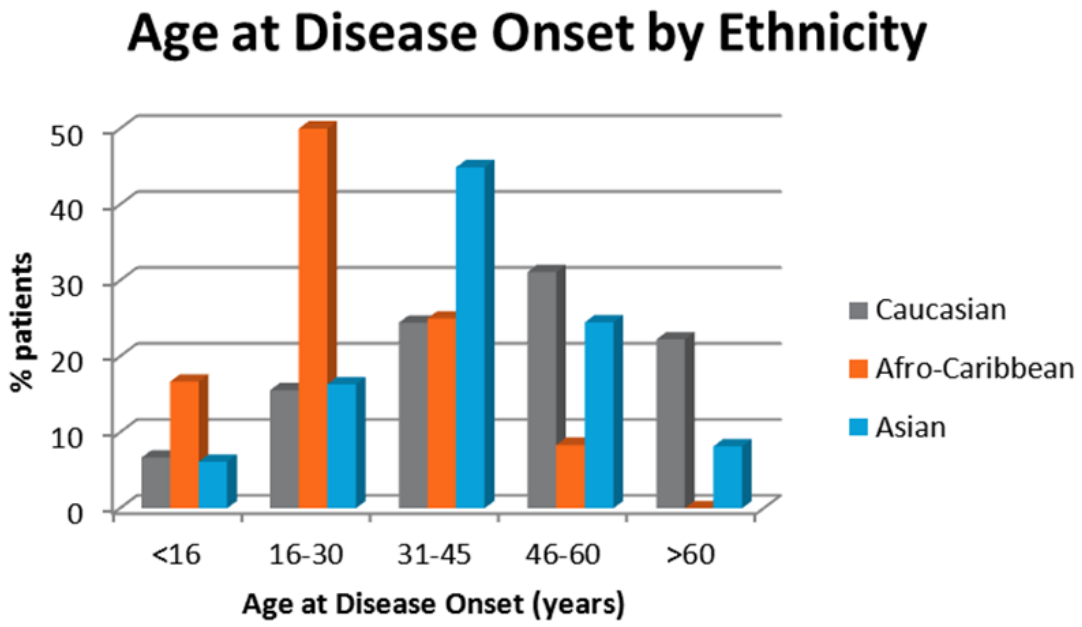


Figure 3.6. Distribution of age at disease onset in different ethnic groups. Afro-Caribbean patients had a significantly younger mean age at disease onset (28.0 ± 13.1 years) than Caucasians (44.9 ± 17.2 years; p=0.003) or Japanese Asians (40.1 ± 13.55; p=0.008). Whilst mean age at disease onset did not differ significantly between Caucasians and Japanese Asians (p=0.159), the Caucasian group had a higher proportion of older onset patients.

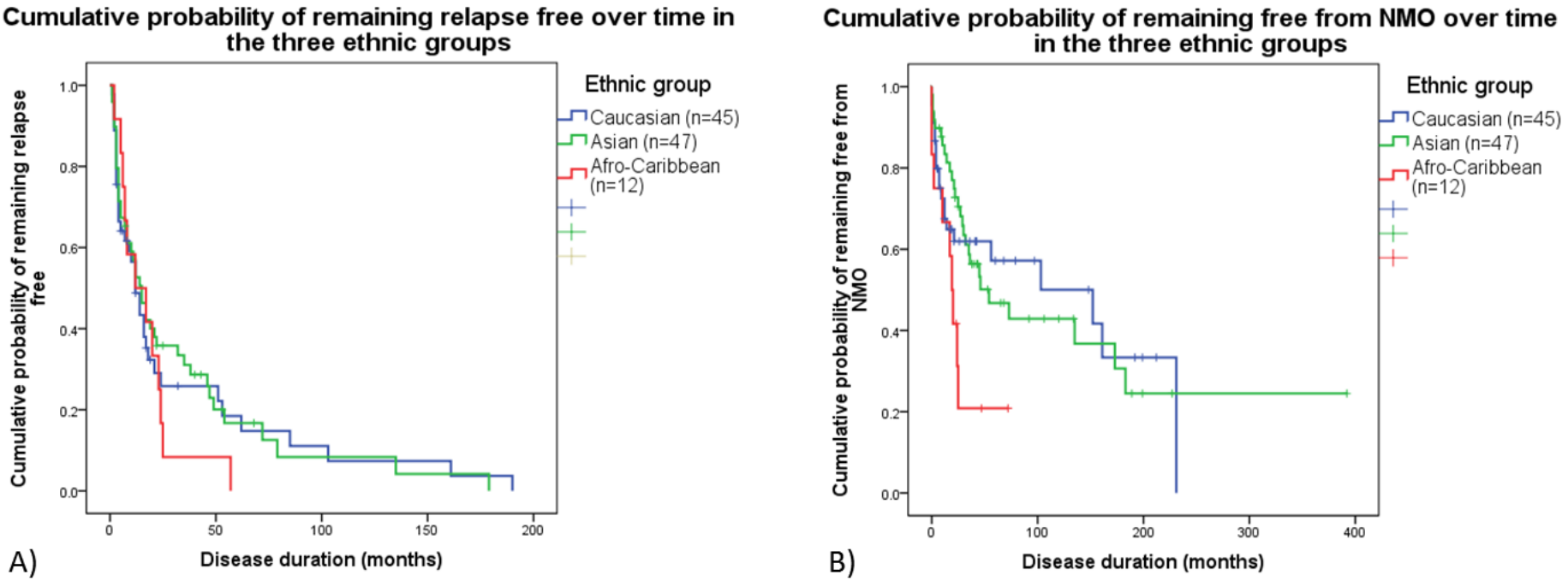


Figure 3.7. Risk of relapse and conversion to NMO over time depending on ethnicity. Kaplan-Meier curves showing cumulative probability of remaining free from relapse (A) or free from progression to NMO (B) over time depending on ethnicity. A) Time to first relapse was not significantly different between ethnicities; B) Afro-Caribbean patients had a greater probability of developing NMO over time than Asian patients (HR 2.69; 95% CI 1.21 to 5.96; p=0.011). The difference between Afro-Caribbean and Caucasian patients did not reach statistical significance (HR 1.47; 95% CI 0.98 to 2.2; p=0.064).

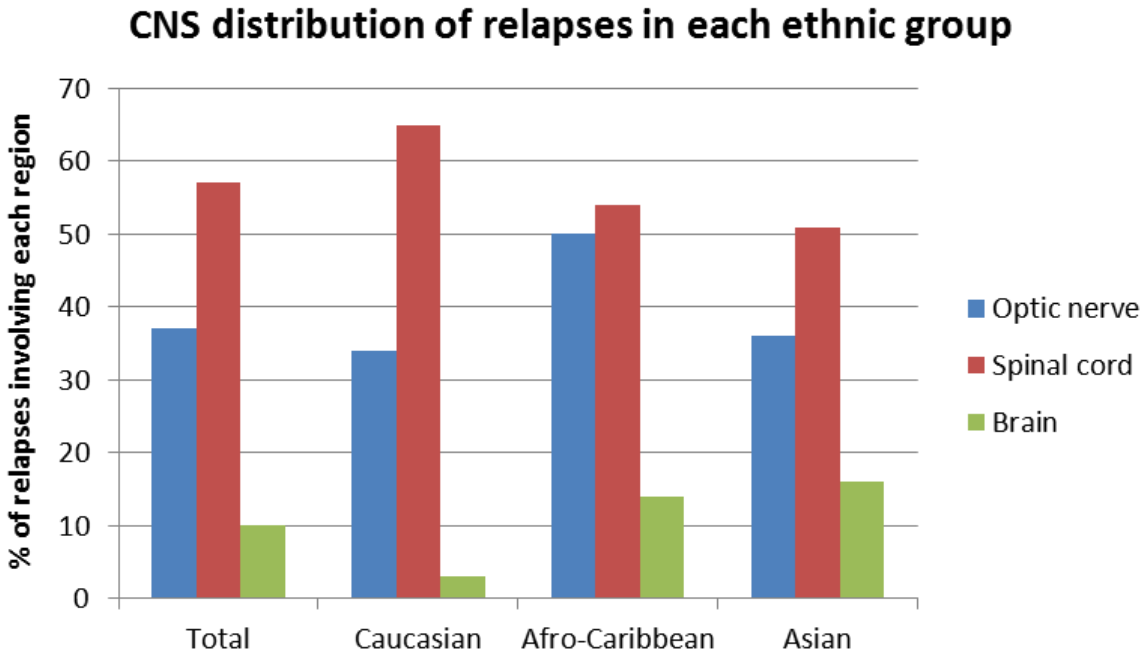


Figure 3.8. CNS distribution of relapses in different ethnic groups. Relapses in Caucasians most commonly involved the spinal cord, whilst only one third affected the optic nerve. Relapses involving the brain were rare. In Afro-Caribbean patients, the optic nerves were involved in half of all relapses and the brain was involved in over 10%. Relapses involving the brain were also relatively common in Japanese Asian patients.

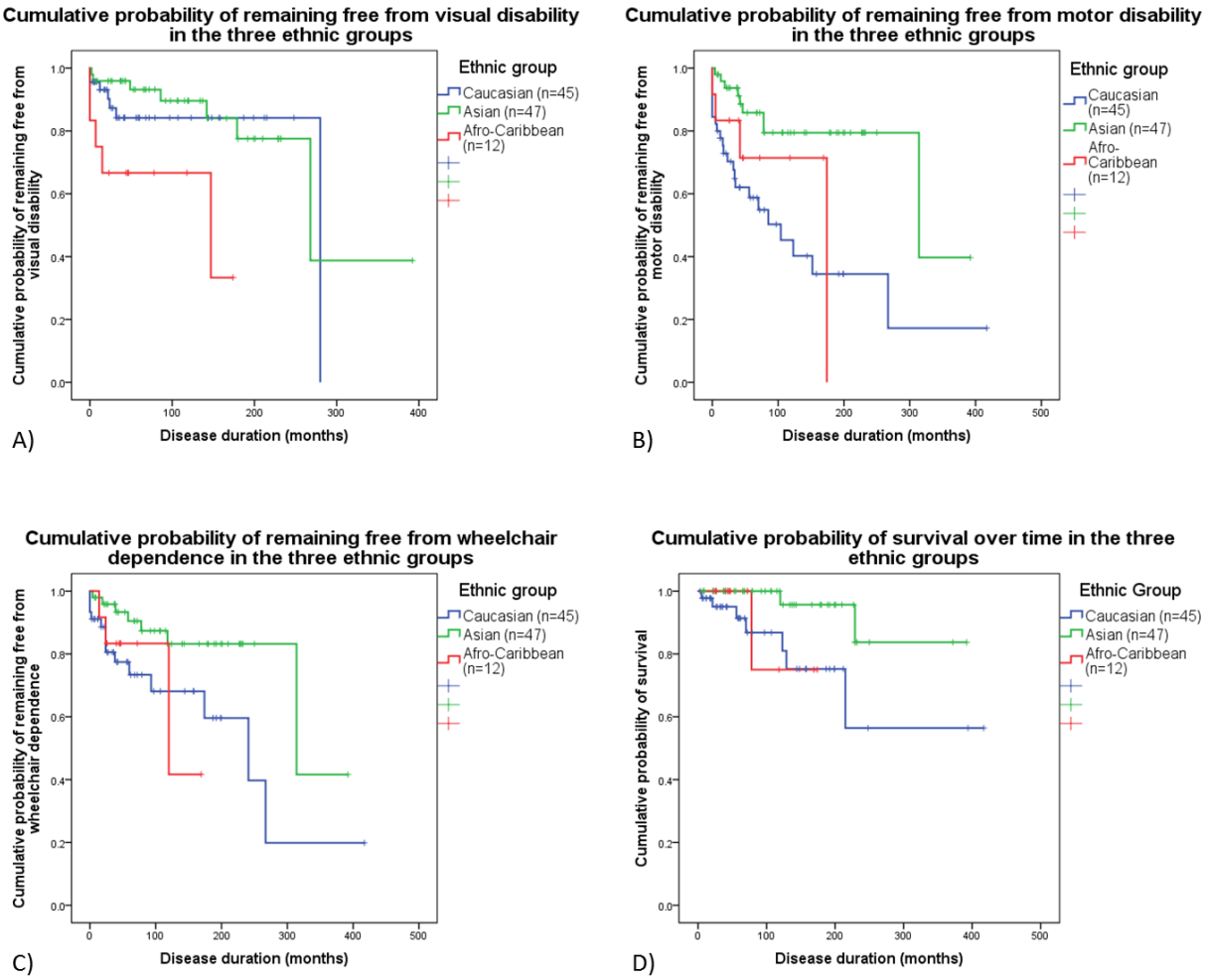


Figure 3.9. Disability outcomes depending on ethnicity. Kaplan-Meier analysis showing cumulative probability over time of reaching disability end-points depending on ethnicity. A) Patients of Afro-Caribbean ethnicity had a significantly higher chance of developing visual disability (best eye worse than 6/36) than Caucasian (42% vs. 16%; HR 1.83; 95% CI 1.01 to 3.31; p=0.046) or Asian patients (12%; HR 3.69; 95% CI 1.139 to 12.048; p=0.030). B) When compared to Asian patients, Caucasian patients were significantly more likely to become motor disabled (inability to walk >100 metres unaided) (49% vs. 18%; HR 3.85; 95% CI 1.8 to 8.4; p=0.001). Afro-Caribbean patients had an intermediate chance of motor disability, which was not significantly different from Caucasians or Asians. C) Caucasian patients were significantly more likely to become wheelchair dependent than Asians (31% vs. 15%; HR 2.819; 95% CI 1.132 to 7.018; p=0.026). Afro-Caribbean patients had an intermediate likelihood of becoming wheelchair dependent. D) Asian patients were significantly less likely to die than Caucasians (4% vs. 16%; HR 0.177; 95% CI 0.036 to 0.860; p=0.032).

more likely to reach motor disability endpoints compared to the Japanese group (Figure 3.9B and 3.9C). Afro-Caribbean patients had an intermediate likelihood of developing motor disability.

The Japanese patients were less likely to die than Caucasians (Figure 3.9D) but there were no differences between other groups.

3.4 Discussion

The majority of previous clinical NMO studies have included AQP4-Ab seronegative patients, or were published prior to the availability of AQP4-Ab assays. A few studies have sub-analysed small groups of AQP4-Ab positive NMO patients within their cohorts but did not include patients with atypical or limited NMO phenotypes (Table 3.4) (Bizzoco, Lolli et al, 2009; Adoni, Lino et al, 2010; Akman-Demir, Tüzün et al, 2011). Only one previous study has described outcomes in an AQP4-Ab positive cohort of NMO and NMOSD patients (Nagaishi, Takagi et al, 2011). ARR, final EDSS, and the proportion of patients developing visual impairment by last follow-up were noted in this Japanese cohort, but prognostic features were not investigated. Several studies have looked at prognostic markers in cohorts that included a large proportion of AQP4-Ab seronegative patients or where AQP4-Ab antibody status was not determined. These studies are summarised in Table 3.5. We are the first to report the effect of ethnicity, treatment and other baseline prognostic factors on time to visual and motor disability outcomes in two AQP4-Ab positive populations (Kitley, Leite et al, 2012).

The two cohorts, in line with previously published data, showed a marked female predominance. The mean age at disease onset was similar to previous studies with the exception of some that included a high proportion of AQP4-Ab seronegative patients and found a younger mean age at disease onset (Collongues, Mariginier et al, 2010; Akman-Demir, Tüzün et al, 2011). This may be because AQP4-Ab seronegative patients tend to be younger than those with AQP4-Ab mediated disease (Nagaishi, Takagi et al, 2011). Afro-Caribbean patients were found to have a significantly younger age at disease onset than Caucasian or Asian patients and this may explain the younger age at onset reported in previous studies of predominantly Afro-Caribbean patients (Cabre,

Table 3.4. Studies detailing demographic and clinical features of AQP4-Ab positive NMO/NMOSD patients

Study	N	FU (years)	Ethnicity	Mean onset age (years)	Onset attack	Disease course	Time to 1st relapse (months)	Time to NMO (months)	% developing NMO	Mean ARR
Current	106	6.3	46% Asian 42% Caucasian 8% Afro-Caribbean	41	42% ON 42% LETM 5% Brain/ brainstem 4% ON & TM	86% RR 14% monophasic	14	54 (17 considering only those who developed NMO)	53	0.82
Akman-Demir, Tüzün et al, 2011	21	8.4	All Turkish; not detailed	33	67% ON 24% LETM 10% ON & TM	95% RR 5% monophasic	N/A	24	All by definition	N/A
Nagaishi, Takagi et al, 2011	583	4.5	All Japanese; not detailed	44	49% ON 46% TM 17% Brain/ brainstem	N/A	N/A	N/A	N/A	1.4
Adoni, Lino et al, 2010	18	7.4	67% Afro-Caribbean 28% Caucasian 5% Asian	24	72% ON 28% TM	All RR by definition	15	N/A	All by definition	N/A
Bizzoco, Lolli et al, 2009	10	10.6	All Italian; not detailed	37	N/A	N/A	N/A	N/A	N/A	N/A

N = number; FU = follow up; NMO = neuromyelitis optica; ARR = annualised relapse rate; ON = optic neuritis; TM = transverse myelitis; LETM = longitudinally extensive transverse myelitis; RR = relapsing remitting; N/A = not assessed.

Table 3.5. Studies reporting predictors of disability outcomes and mortality in NMO

Study	N	% AQP4-Ab +ve	Predictors of disability	Predictors of mortality
Wingerchuk, Weinshenker et al, 2003	80	N/A	N/A	Older age at disease onset, history of autoimmune disease, bilateral ON and higher attack frequency in the first two years were associated with mortality on univariate analysis. In multivariate analysis, history of autoimmune disease, degree of motor recovery after the onset attack and relapse frequency in the first two years of disease were significant
Ghezzi, Bergamaschi et al, 2004	46	N/A	Probability of reaching EDSS 6 was higher in older onset patients, those with a shorter interval between their first and second attacks and those with a higher relapse rate	N/A
Bichuetti, Oliveira et al, 2009	41	41	Patients who reached EDSS 6 had higher EDSS at first presentation than those who did not. Onset age, gender, relapse rate and disease duration did not correlate with assignment of EDSS 6	N/A
Cabre, González-Quevedo et al, 2009	32	32	N/A	Blindness after the first attack, higher relapse rate in the first year of disease, first relapse within one year of disease onset, lack of recovery after the first attack and sphincter signs at onset were associated with shorter time to death. Sex, ethnicity, age at onset and type of onset attack were not predictive of mortality
Collongues, Marignier et al, 2010	125	54	Patients presenting with simultaneous ON and TM developed visual failure earlier than those presenting with either TM or ON. Higher number of brain lesions was predictive of shorter time to visual loss Topography of first attack was not predictive of time to assignment of EDSS scores Late onset of disease, short interval between first two attacks and high number of attacks in the first year of disease were predictive of time to EDSS in univariate but not multivariate analysis	N/A

N = number; N/A = not assessed; AQP4-Ab = aquaporin-4 antibody; +ve = positive; ON = optic neuritis; TM = transverse myelitis; EDSS = Expanded Disability Status Scale.

Heinzlef et al, 2001; Merle, Olindo et al, 2007) although in addition these reports also included seronegative patients.

Patients with ON as their onset attack were significantly younger than LETM-onset patients. All but one of the paediatric-onset patients presented with ON, whilst two-thirds of late-onset patients (>50 years) presented with LETM. This observation may suggest age-dependent anatomical susceptibility differences or differences in AQP4-Ab accessibility of the target organs. ON as a younger patient phenomenon is notable in MS as well (Scalfari, Neuhaus et al, 2011), so the optic nerves may be generally more vulnerable to inflammatory insults in younger age groups.

A 'classic' Devic's syndrome [as originally described (Devic, 1894)] with simultaneous ON and TM as the onset attack was rare in both cohorts (5% and 2% in the UK and Japanese cohorts respectively). By contrast, higher rates have been noted when AQP4-Ab seronegative patients are included in cohorts (Rivera, Kurtzke et al, 2008; Bichuetti, Oliveira et al, 2009; Collongues, Marignier et al, 2010; Akman-Demir, Tüzün et al, 2011). It is possible that as yet unidentified antibodies are responsible for some cases of seronegative NMO presenting in this manner. Our current study suggests that 'classic' Devic's syndrome is a relatively rare presentation of AQP4-Ab mediated NMO and alternative diagnoses such as acute disseminated encephalomyelitis (ADEM) should be considered when patients present in this manner and have no detectable AQP4-Ab.

The group, of whom the majority were not started on immunosuppression after their onset attack, had a median time to first relapse of 14 months, which is similar to a small subgroup of AQP4-Ab positive NMO patients reported recently (Adoni, Lino et al, 2010). Overall, 49% of patients experienced a second attack within one year of the onset event, 70% within two years. This high risk of relapse is in keeping with other studies of AQP4-Ab positive LETM and ON patients (Weinshenker, Wingerchuk et al, 2006; Matiello, Lennon et al, 2008; Collongues, Marignier et al, 2011) and supports the view that AQP4-Ab positive patients should be offered early immunosuppressive therapy in a bid to prevent subsequent relapses. Early immunosuppressant treatment in our cohorts appeared to delay time to first relapse and ARR were significantly lower

after starting treatment. Twelve of 23 patients treated before their first relapse never had another relapse, though it should be noted that follow-up time was relatively short in such monophasic patients and it is possible that some of these would have developed relapsing disease with time. Interestingly, four patients, all with ON as their onset attack, had an interval (off treatment) between their onset attack and first relapse of greater than 10 years. Two of these patients subsequently developed permanent bilateral visual disability. Fifteen patients (14%) had only a single attack of CNS inflammation over the study follow-up period and were said to have monophasic disease. This is likely a reflection of the relatively short follow-up time in this group together with the fact that long-term immunosuppression was commenced early (within four months of the onset attack in all patients for whom treatment data was available (n=12) with the exception of one patient). Overall, the mean ARR was found to be 0.82, but decreased over time, particularly after the first two years. This may be explained by regression to the mean, treatment effects, or burning out of disease activity with time. In support of treatment effects being a contributing factor, 50% of patients in the group had no relapses after commencing treatment. Due to widespread acceptance that immunosuppressive agents are effective in preventing NMO relapses, it is unlikely that any true natural history studies will be performed in NMO in the future. At present it is not known whether it is safe to withdraw treatment after several years of disease inactivity in some patients.

Median time to developing NMO (at least one attack of both ON and TM) was relatively long in our cohort (54 months) and six patients experienced a delay of greater than 10 years. Patients and clinicians must therefore remain vigilant to the possibility of involvement of other parts of the nervous system even years after disease onset. Patients presenting with ON had a higher probability of developing NMO with time than those presenting with LETM. However, it may be that AQP4-Ab mediated monophasic or relapsing ON is under-recognised as many may not present to neurology services and be screened for AQP4-Ab, whereas those with LETM are more likely to be tested. For the same reason, prophylactic immunosuppressant therapy after a first LETM attack

is likely to be more common than after a single ON attack. Therefore, diagnostic and treatment differences between these two groups of patients may influence and confound disease course.

In our cohort, 47% of patients did not develop NMO according to the 2006 diagnostic criteria (Wingerchuk, Lennon et al, 2006). A significant proportion experienced relapsing LETM or relapsing ON only and a small group had brain/brainstem disease. Although it is likely that some patients would have developed NMO over a longer follow-up period, these findings demonstrate that AQP4-Ab mediated disease is not synonymous with the classical description of NMO. This raises the question of whether these patients should be incorporated into NMO diagnostic criteria, and whether we should be moving towards defining NMO/NMOSD more by AQP4-Ab status. AQP4 channelopathy disease may classify a single disease pathology and be more useful, although highly specific and sensitive assays comparable across sites would be a prerequisite.

Morbidity and mortality in our cohorts were substantial, and after median disease duration of 75 months, around one fifth had developed permanent bilateral visual disability (visual acuity worse than 6/36 in best eye), one third permanent motor disability (unable to walk >100 metres unaided) and of these nearly one quarter had become wheelchair dependent by last follow-up. Nine per cent died. Within our study, ethnicity and age at disease onset appeared to be important predictors of visual and motor disability and mortality. Additionally, male gender seemed to be predictive of worse visual outcomes.

Young-onset patients had a higher probability of developing visual disability whilst late-onset patients had a greater likelihood of developing motor disability. Fifty per cent of paediatric-onset patients (four of eight patients) reached the visual disability endpoint, whilst none developed permanent motor disability (despite 75% experiencing attacks of LETM). By contrast, only two of 29 late-onset patients developed visual disability whilst 18 reached the motor disability endpoint. As discussed previously, onset age influenced type of onset symptom and so young patients had a ‘head start’ over older-onset patients for developing visual disability as they were more likely to present with ON, and vice versa for motor disability. However, this can only partly explain the

differences in disability outcomes, as late-onset patients had an equally high risk of developing motor disability whether they presented with ON or with LETM. This suggests that there may be a lower capacity for spinal cord recovery with increasing age which could explain the worse motor outcomes in older patients presenting with LETM. However, it is less clear why older patients presenting with ON (where the AQP4-Ab have already penetrated the optic nerve blood brain barrier) had a lower risk of developing visual disability than young-onset patients. Older age at disease onset was also predictive of mortality. This may be because older age was associated with motor disability, which predisposes to life-threatening complications such as bronchopneumonia.

There was a trend towards the UK cohort having a greater likelihood of developing visual disability with time than the Japanese cohort. This appeared to be driven by poor visual outcomes in the Afro-Caribbean UK patients, who had a significantly greater likelihood of developing visual disability with time than Caucasian patients or the Japanese cohort. In one previous study that included AQP4-Ab seronegative patients, visual impairment was found to be more severe and more frequent in Afro-Brazilian patients compared to white Brazilians (Papais-Alvarenga, Miranda-Santos et al, 2002). Thus, although the number of Afro-Caribbean patients in our study was small (n=12), thereby reducing the reliability of our results, it appears that Afro-Caribbean ethnicity may predict more severe optic nerve involvement in NMO/NMOSD. This may be because Afro-Caribbean patients tended to present at a younger age, with onset attacks that included ON, both of which were predictors of poor visual outcomes. Additionally, subsequent attacks were also more likely to involve the optic nerves than in other ethnic groups. Because of the small numbers in this group (n=12) it was not possible to correct for these confounders in the statistical analysis. However, it is an interesting observation whether Afro-Caribbean ethnicity results in worse visual outcomes *per se*, or whether Afro-Caribbean ethnicity predicts earlier age at disease onset and propensity for attacks to involve the optic nerves, which in turn predicts poor visual outcomes. Ethnicity also appeared to affect motor outcomes, with the Caucasian patients having a significantly higher probability of developing motor disability or wheelchair dependence over time than the Japanese cohort. Afro-Caribbean patients had an intermediate probability of

developing motor disability. Caucasian patients also had a higher likelihood of mortality over time than the Japanese group.

Only one other study (of only Japanese patients; Nagaishi, Takagi et al, 2011) has described visual and motor outcomes in AQP4-Ab seropositive NMO/NMOSD patients. In this study, 16.2% patients were reported as blind and 28.6% severely visually impaired after median disease duration of 4.5 years. The higher rate than the 12% seen in our Japanese group (after a longer median follow-up time in our study of 6.25 years) could be because Nagaishi included those with both unilateral and bilateral visual impairment, whereas we used bilateral visual impairment as our visual endpoint. The reason for this was because bilateral visual disability was considered to be more functionally relevant. Mean EDSS at last follow-up in the Nagaishi cohort was 5.6, but no details were provided on the proportion of patients requiring walking aids or developing wheelchair dependence and so no comparison can be made with the current study.

Although the Japanese cohort had significantly longer follow-up than the UK cohort, the UK cohort appeared to have more severe disease. In addition to the differences seen in disability outcomes between the two populations, the UK cohort also had more severe onset relapses and a higher mean ARR. One possibility for the differences in motor and visual outcomes between the groups is the different distribution of age at disease onset (Figure 3.7). Although the mean age at disease onset did not differ between Caucasian and Japanese patients, there were a greater proportion of late-onset patients within the Caucasian group. Since older age was predictive of motor disability and mortality, the differences noted in motor disability and mortality outcomes between the two groups may be explained by the fact that the Japanese cohort contained less 'at risk' older patients. Of course, this difference in age distribution may be genetically driven.

Treatment strategies used in the two populations as an explanation of the differences noted in disability outcomes need to be considered. However, although there were individual variations in immunosuppression regimes, all sites started treatment with high dose corticosteroids, tapering over several months to a similar maintenance dose. Additionally, most UK patients and some

Japanese patients were also prescribed azathioprine 2.5mg/kg. Fourteen patients (thirteen UK, one Japan), who were diagnosed during disease remission, commenced long-term immunosuppression with azathioprine alone, but only one patient experienced a relapse within the first six months of treatment. Acute relapse regimes in the two cohorts were also similar (intravenous methylprednisolone followed by an oral corticosteroid taper) although the influence of short delays in treatment cannot be assessed.

As these cohorts were not population based, different referral biases between the groups cannot be excluded, although we are unable to postulate a systematic bias to explain our findings. Additionally, the differences noted between the Caucasian and Afro-Caribbean UK patients could not be explained by different cohort referral patterns. This suggests that genetic factors are likely to influence the course and prognosis of NMO/NMOSD. Additionally, environmental factors may also play a role.

One limitation of the study is that all patients were followed in specialist NMO clinics. This might introduce referral and follow-up biases and influence speed of diagnosis and treatment. However, the majority of patients were still from within regional catchment areas. A further potential source of bias is differences in follow-up patterns between centres, particularly between the UK and Japan. Although all three centres follow patients closely and review patients promptly in the context of a potential relapse, slight differences in follow-up patterns as a confounding effect cannot be completely excluded. Additionally, most of the study data were collected retrospectively, which invariably introduces recall bias, particularly regarding timing and severity of relapses and speed and degree of recovery. However, because of the severe nature of the disease, it is unlikely that relapses were missed.

Since all but one patient in the cohort received some form of long term immunosuppression, this is by no means a natural history study. However, it is still important to understand the history of treated NMO/NMOSD as this influences management and counselling of the patients.

Finally, due to the rarity of NMO, there are statistical limitations. Because of the relatively small numbers in the different subgroups, corrections for multiple comparisons and multivariate regression analysis were not performed. Therefore, it is difficult to fully adjust for confounding variables. Where possible, individual groups were analysed separately but small numbers meant this was not possible for all analyses. One needs to be cautious in extrapolating data from one ethnic cohort to another.

In conclusion, the disease course, visual and motor disability outcomes and mortality statistics in 106 AQP4-Ab-positive NMO/NMOSD patients have been described, and the features of a predominantly Caucasian UK cohort have been compared with those of a purely Asian Japanese cohort, and within the UK cohort features have been compared between patients of Caucasian and Afro-Caribbean ethnicity. Important characteristics of the disease course and severity have been described and predictors of time to visual and motor disability have been identified. Such prognostic indicators are helpful for setting up individualised monitoring and treatment regimes. The UK cohort appeared to have more severe disease than the Japanese cohort and within the UK cohort there were important differences between Caucasian and Afro-Caribbean patients. Thus, genetic factors are likely to be important in determining clinical outcomes in AQP4-Ab disease. This has important implications for interpreting clinical NMO studies, since clinical features and outcomes appear not to be generic across populations and may need to be tailored to individual groups. Further studies across ethnic groups are indicated. With increasing recognition of the relevance of AQP4-Ab, earlier treatment and better outcomes are likely in the future.

Chapter 4: AQP4-antibody titres and isoform specificities

4.1 Introduction

There is marked clinical heterogeneity across AQP4-Ab positive NMO/NMOSD patients. Whilst many patients have severe disease with early acquisition of disability, others may have a mild phenotype without accumulating significant relapse-associated disability despite many years of disease (Collongues, Cabre et al, 2011). Additionally, in chapter 3, we showed that a substantial proportion of patients have limited disease phenotypes, with 20% of patients having relapsing LETM and 9% experiencing relapsing ON, without going on to develop the full NMO phenotype. The reasons for this heterogeneity are not clear and make it difficult to predict future outcomes in order to modify treatment strategies. Whilst it is likely that genetic and environmental factors influence disease phenotype and severity, it is also possible that immunological factors are involved.

The two most abundant AQP4 isoforms in humans are M1 and M23. Both isoforms form heterotetramers at the cell surface but the M23 isoform lacks a 22 amino acid N-terminus, which enables these heterotetramers to aggregate into dense clusters called OAPs. Whilst the M1 isoform cannot form OAPs, it can integrate into M23 OAPs to limit their size. Several independent groups have shown *in vitro* that whilst human NMO-IgG binds to both AQP4 isoforms, it has higher affinity for the M23 isoform (Nicchia, Mastrototaro et al, 2009; Mader, Lutterotti et al, 2010; Crane, Lam et al, 2011; Iorio, Fryer et al, 2012). However, a wide range of relative binding to M23 and M1 between patients and between recombinant monoclonal AQP4-Abs from the same patient were recently demonstrated (Crane, Lam et al 2011); the clinical significance of this has not been investigated.

The relative binding of patient antibodies to M23 and M1 may influence disease phenotype. Although the relative expression of M23 and M1 in different parts of the human CNS has not been well characterised, it is possible that expression varies and this may influence which anatomical regions are affected. Additionally, it was recently shown that AQP4-Ab binding to M23 but not

M1 led to complement activation and cell death and therefore it is possible that patients with high levels of M23 antibodies may have more severe disease as a result of enhanced complement activation. Alternatively, it is possible that high M1-Ab levels may inhibit the ability of M1 isoforms to integrate into OAPs, resulting in larger sized OAPs with a greater ability to activate complement, leading to more severe disease.

4.1.1 Aims

The aims of this chapter were to investigate whether there were inter- and intra-patient differences in M23-AQP4 and M1-AQP4 titres and if so, to assess whether these may be of clinical or prognostic importance.

In particular, the aims of this chapter were:

- To confirm previous studies showing that relative binding to M23 and M1 AQP4 differs between patients
- To investigate whether M23 and M1 antibody titres change over time, for example with disease activity
- To investigate whether such differences may be of clinical or prognostic significance

4.2 Technical Approaches

Sera from all AQP4-Ab positive NMO/NMOSD patients known to the Oxford clinical NMO service were collected prospectively at outpatient visits and during relapses (pre and post treatment where possible) from September 2010 to September 2012. Additionally, archived material was available for some patients. Selection of sera for measurement of M23 and M1 titres was based on availability of sera. The earliest available samples from 34 patients were tested, in addition to 44 longitudinal samples from 15 patients.

M23 and M1 antibody titres were measured using FACS analysis of HEK293 cells stably transfected with either the M23 AQP4 isoform or the M1 AQP4 isoform. FACS provides a quantitative score (MFI) reflecting binding of human IgG to AQP4-transfected HEK cells. A

positive score was considered to be a value greater than two s.d. above the mean score of 32 healthy and MS control samples (cut-off score was 21). End-point dilution titres were calculated by running serial doubling dilution samples starting at 1:20; the dilution of the lowest concentration positive sample was taken as the titre. FACS was chosen over CBA because it generates a quantitative objective score for each sample, whereas CBA scores are observer dependent. Each sample was tested at least twice and experiments were performed blinded to clinical status.

4.3 Results

4.3.1 Commercial AQP4-Ab binding to M1 and M23 AQP4 isoforms

A commercial AQP4-Ab, which binds to the intracellular C-terminus common to both M23 and M1 AQP4 isoforms was found to bind to both M23 and M1 stably transfected HEK293 cells. End-point dilution titres for the commercial AQP4-Ab were the same for both M23 and M1 transfected cells, suggesting a similar level of binding to the two isoforms. Because the commercial AQP4-Ab binds to an intracellular epitope, a CBA was used to detect its binding, since permeabilised cells were not suitable for FACS analysis.

4.3.2 FACS can detect binding of human IgG to both M23 and M1 transfected cell-lines

Human-IgG from sera known to be positive for AQP4-Ab on the Oxford diagnostic CBA was found to bind to both M23 and M1 stably transfected cells and could be detected by FACS, whilst healthy control sera did not bind to M23 or M1 stably transfected cells. Thus, FACS of stably transfected cell-lines was a suitable method to use for measuring M23 and M1 titres.

4.3.3 Binding curves of AQP4-Ab positive samples

To help establish how best to compare titres across and within patients, FACS was performed on serial doubling dilutions of serum samples from 10 patients and binding curves were plotted. This revealed different binding curves for different patients, and sometimes different binding curves for

different samples from the same patient. As would be expected, most samples exhibited a sigmoidal binding curve, with a linear portion that became non-linear once FACS scores became very low (Figure 4.1A). The linear portion of the curve was sometimes preceded by a plateau phase. Surprisingly, however, some patient samples had a bell-shaped binding curve (Figure 4.1B), whereby at very high concentrations of sera, FACS scores were actually lower than at lower concentrations. These bell-shaped curves followed a linear course after reaching their plateau phase, which was to be expected. Some samples exhibited a long plateau phase on this bell-shape whilst others were more 'pyramid-shaped'.

The reasons for the bell-shape of some binding curves are not entirely clear. It may be because at high antibody concentrations, competition is so fierce for antigen binding that antibodies bind only monovalently and 'fall off' when cells are centrifuged or, probably more likely, cells with bound patient IgG undergo cell death and residual cell fragments are not detected because only viable cells are gated and analysed.

4.3.4 Validation of FACS assay for measuring antibody titres

FACS could be used in several ways to compare titres between samples and between patients:

- Comparison of maximum FACS scores i.e. MFI when serial dilutions are run
- Comparison of FACS score i.e. MFI at a chosen dilution
- End-point dilution values

First, it was determined which of these methods would be most reliable to compare samples.

4.3.4.1 Comparing peak FACS scores

Running the same sample on three different assays revealed that there was significant inter-assay variability in maximum FACS score i.e. peak MFI, particularly when peak scores were high (MFI >1000) (Figure 4.2). Thus, comparing peak MFI values as a way of comparing titres between samples and between patients was deemed to have insufficient reliability.

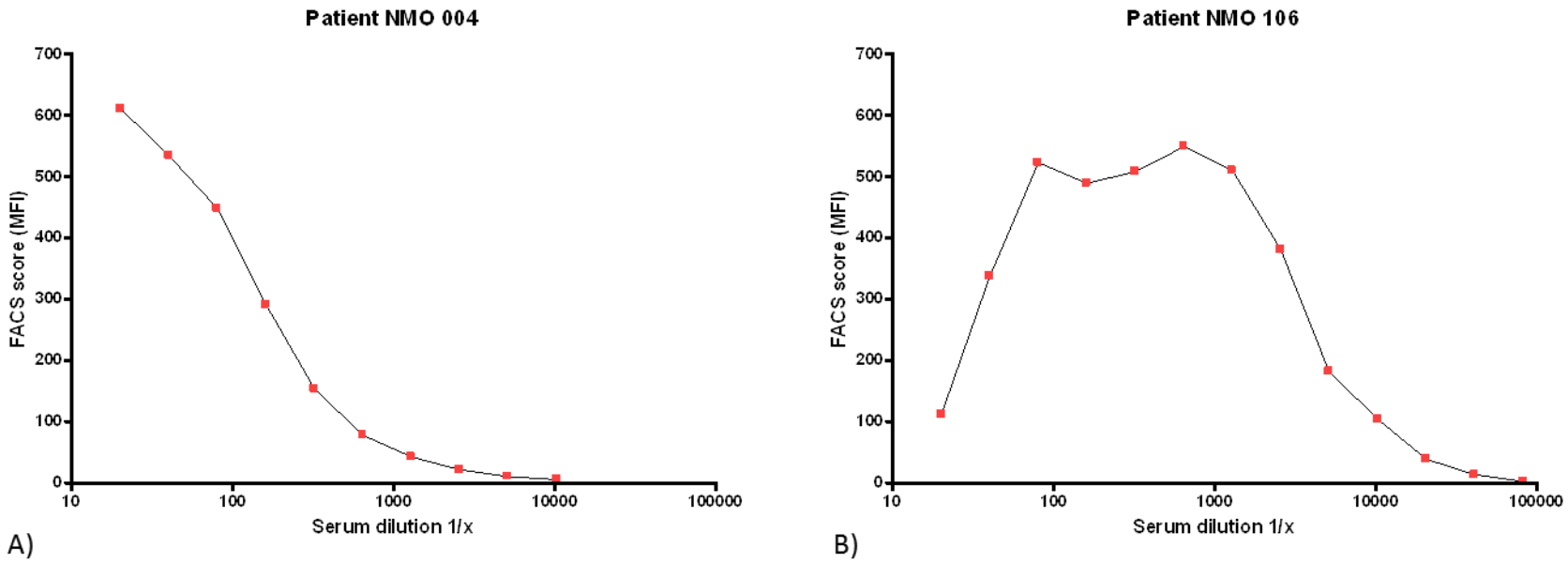


Figure 4.1. Examples of binding curves of doubling dilutions of NMO sera. Doubling serial dilutions of patient serum samples were performed starting at a 1:20 dilution and each sample was analysed by flow cytometry (FACS); dilutions were plotted against FACS scores [median fluorescence intensity (MFI)] to create a binding curve. Most patient serum samples exhibited a sigmoidal binding curve (A) but some generated a bell-shaped curve (B). Note log10 scale on x axis.

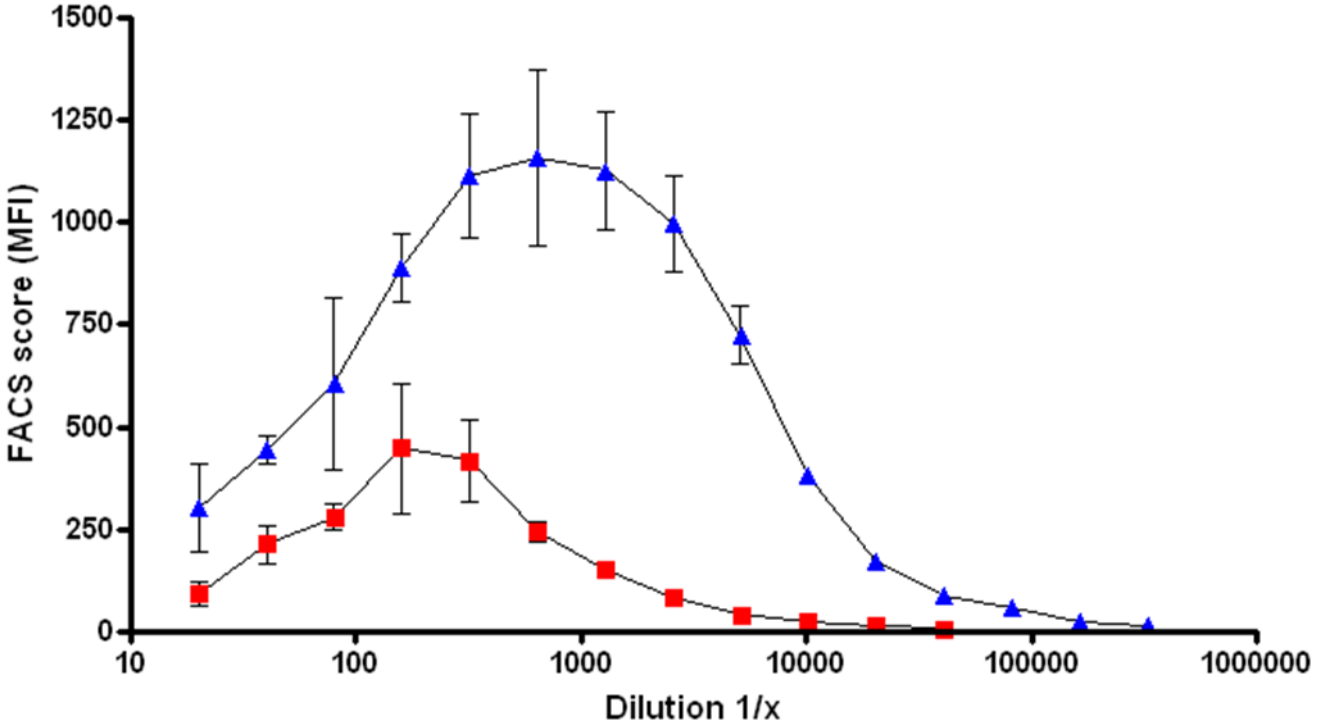


Figure 4.2. Inter-assay variability in peak median fluorescence intensity. There was significant inter-assay variability in maximum FACS score (peak MFI), making comparisons of maximum FACS scores a sub-optimal method for comparing titres between samples. Error bars represent inter-assay variability from three experiments. Blue triangles represent scores for M23 binding and red squares represent scores for M1 binding for the same sample. Note log10 scale on x axis.

4.3.4.2 Comparing FACS scores at a set dilution

Comparing MFI values at a set dilution of sera is attractive as a method of comparing samples because it is quick and easy. However, because the shape of binding curves was shown to differ between patients, and even between different samples from the same patient, it was felt that using FACS scores at a given dilution to compare 'titres' within and across patients may not provide representative data. Because of the bell-shaped binding curve for some samples, measuring FACS scores at a set dilution e.g. 1:100 could have provided a score on the rising slope of the bell curve and be misinterpreted as indicating low antibody levels rather than the true value which should be measured from the descending slope (Figure 4.3).

4.3.4.3 End-point dilution values are reproducible despite variations in peak MFI

End-point dilutions are time consuming but probably give the most information about a sample as the full shape of the binding curve can be seen. It was shown that although there was inter-assay variation in peak MFI, this did not influence end-point dilution titres (Figure 4.4). Of 85 patient samples run on two different assays, end-point dilution titres were within a single doubling dilution on repeat assaying for 81 pairs (95%) of all samples run.

Overall, end-point dilutions were felt to give the most information and be the most reproducible way of comparing titres between samples. End-point dilutions were thus performed on all subsequent samples.

4.3.4.4 Correlation between FACS and CBA end-point dilution titres

To ensure validity of the FACS assay for measuring end-point dilution titres, M23 end-point dilutions were also measured by CBA on 41 samples from 18 patients. CBA end-point dilution titres were measured by Dr Mark Woodhall. There was good correlation between end-point dilution titres measured using the two techniques (Figure 4.5; $r=0.802$; $p<0.0001$).

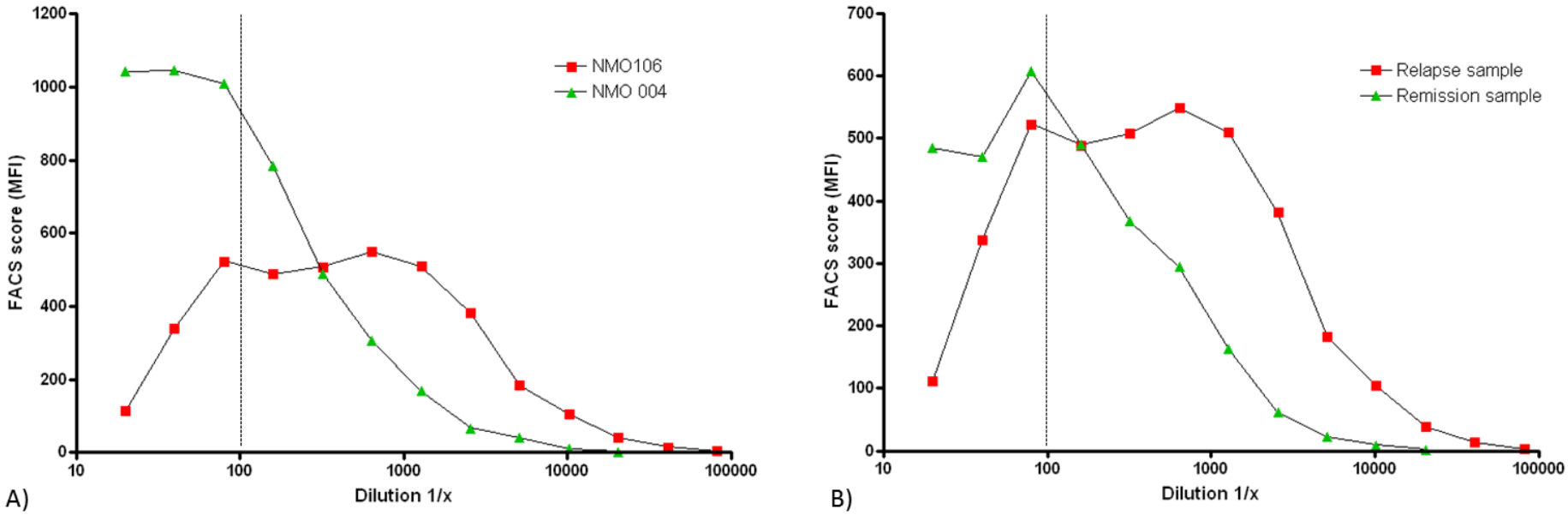


Figure 4.3. Measuring median fluorescence intensity at a set dilution. A higher FACS score i.e. median fluorescence intensity (MFI) at a given dilution is not necessarily indicative of higher antibody titres. A) Binding curves of two samples from two different patients. The sample from patient NMO 004 (green triangles) gives a higher MFI at a concentration of 1:100 than the sample from patient NMO 106 (red squares) but the sample from NMO 106 had a higher end-point dilution titre. B) Binding curves of two samples from the same patient, one during relapse (red squares) and one during remission (green triangles). The relapse sample has a higher concentration of antibodies as it remains positive on FACS at much lower serum concentrations than the remission sample, but if FACS scores were compared at a standard dilution of 1:100, the remission sample would have given a higher value. Note log10 scale on x axis.

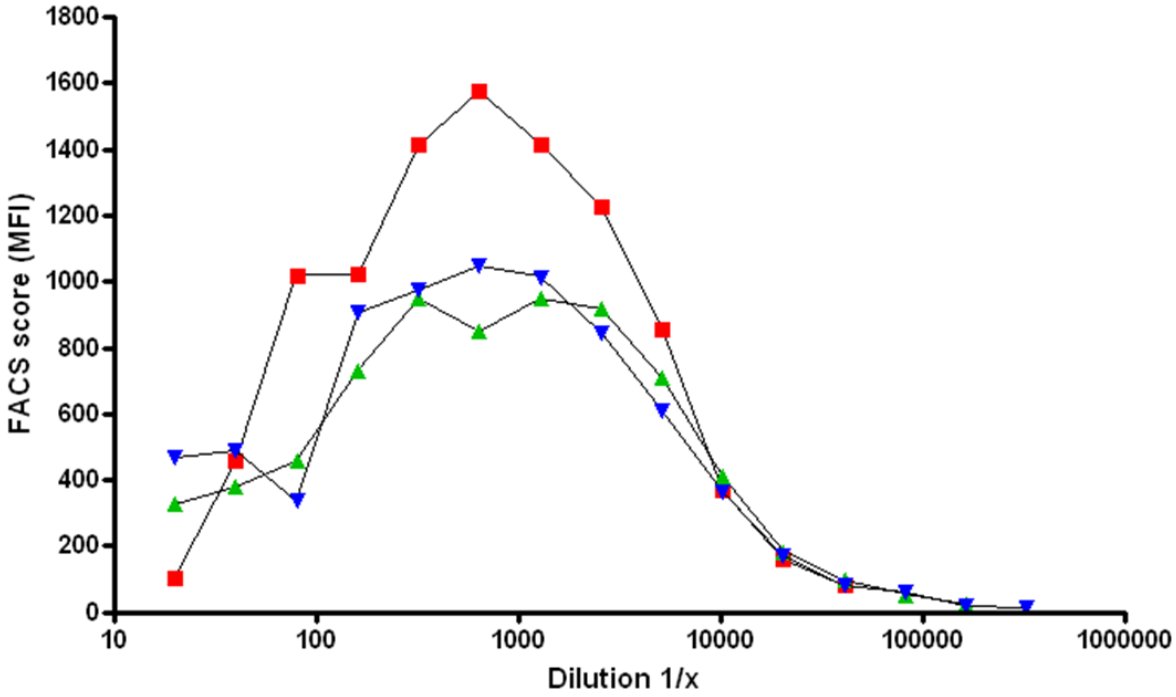


Figure 4.4. Relationship between median fluorescence intensity and end-point dilution titre. The graph shows binding curves from a single sample from patient NMO 017 run on three different assays. Although there was inter-assay variability in peak median fluorescence intensity (MFI), the end-point dilution titres were identical i.e. peak MFI did not predict end-point dilution. Note log10 scale on x axis.

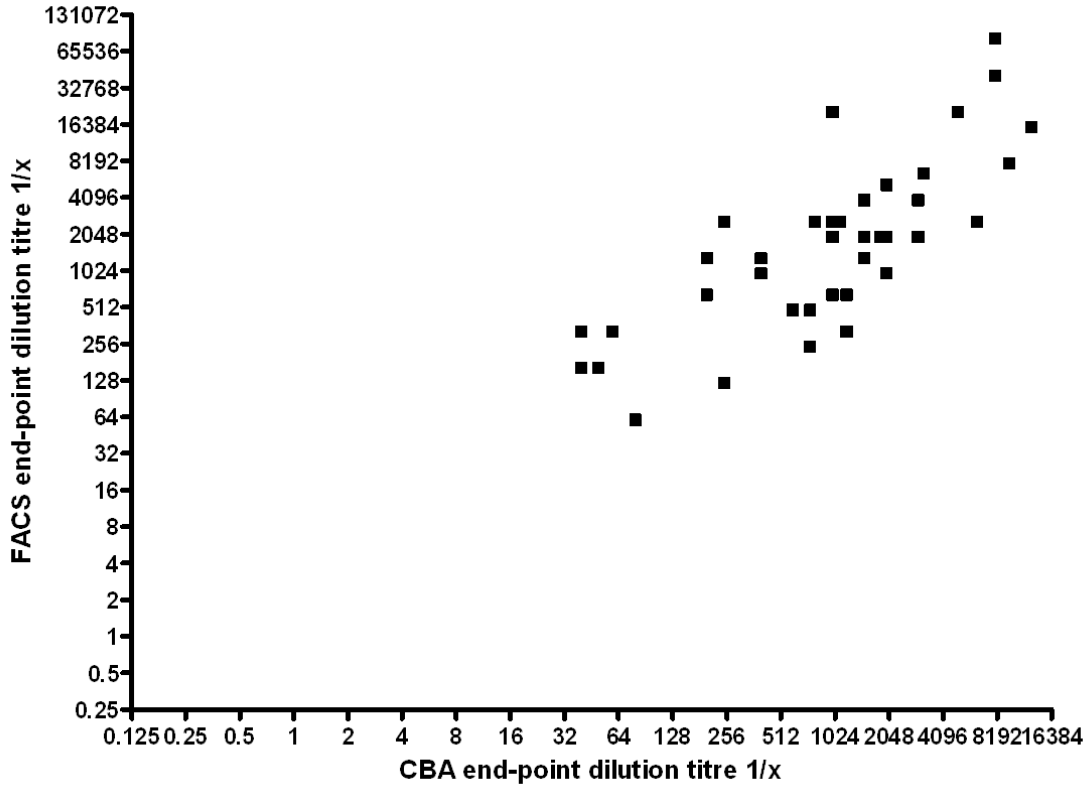


Figure 4.5. Correlation between FACS and CBA end-point dilution titres. Flow cytometry (FACS) and cell-based assay (CBA) end-point dilution titres were measured on 41 samples from 18 patients. There was good correlation between the two techniques ($r=0.802$; $p<0.0001$). Note log2 scale on x and y axes.

4.3.5 Relative M1 and M23 isoform binding specificity

4.3.5.1 Frequency of detectable M23 and M1 antibodies

Having shown that end-point dilution titres were the best way to compare samples, M23 and M1 AQP4-Ab end-point dilution titres were determined retrospectively in 78 samples from 34 clinically well-characterised NMO/NMOSD patients (26 relapse samples, 52 remission samples). M23 antibody titres were higher than M1 titres in 76 of the 78 samples; two samples (from two different patients) had equal titres. M1 antibodies were detectable in 19 of 26 relapse samples and 33 of 52 remission samples, whilst M23 antibodies were undetectable in only two remission samples from a single patient and all patients had M23 antibodies detectable in at least one sample.

4.3.5.2 Relationship between M23 and M1 antibody titres

There was no clear relationship between M23-Ab and M1-Ab titres (Figure 4.6) and the M23:M1 ratio varied between patients (range 192:1 to 1:1) and was not predicted by end-point dilutions of either isoform. In general, M1-Ab were absent only when patients had low M23-Ab titres, though no threshold cut-off could be identified. The majority of samples with M23-Ab end-point dilutions of <500 had no detectable antibodies to M1 (15 of 21 samples, 71%), although two samples still had detectable M1-Ab with M23-Ab levels as low as 160. Conversely, two patients had no detectable M1-Ab when M23-Ab titres were >2500; the highest M23-Ab titre without detectable M1-Ab was 5120. One patient (NMO 007) had no M1-Ab detectable in any of 11 samples, despite six of these being taken during relapse.

4.3.5.3 Pre-adsorption

To investigate whether antibodies against M23 may be different from those directed against M1, pre-adsorption experiments were performed. Serum samples were used from three patients with identical M23 end-point dilutions – patient NMO 007 with no detectable M1 antibodies, patient NMO 145 with equal M23 and M1 end-point dilution titres, and patient NMO 004 with an M23:M1 end-point dilution ratio of 2:1. Sera were pre-adsorbed on either M23-transfected, M1-

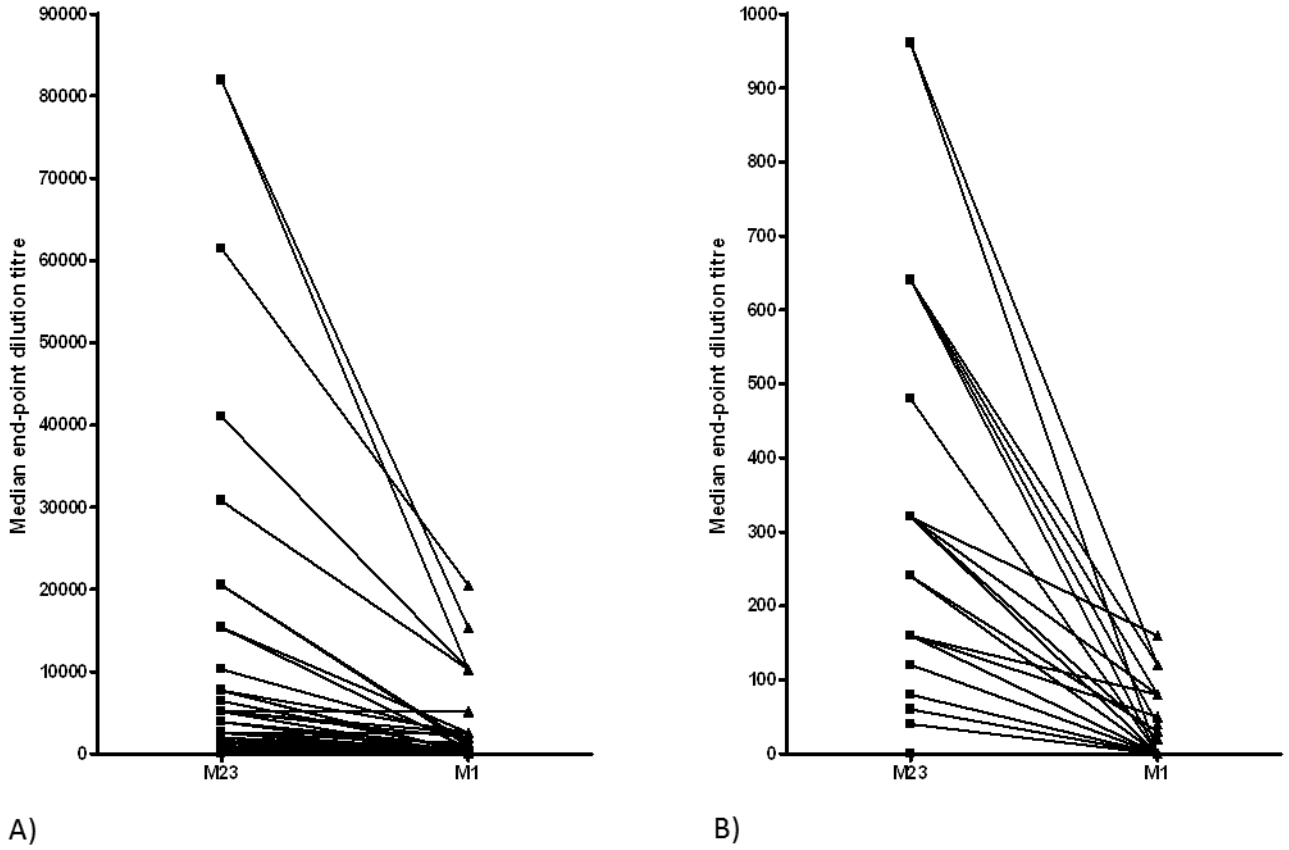


Figure 4.6. Relationship between M23-Ab and M1-Ab titres. M1-Ab titre was not predicted by M23-Ab titre. A) Paired M23-Ab and M1-Ab titres from all 78 patient samples and B) Paired M23-Ab and M1-Ab titres plotted only from samples with an M23-Ab titre of <1000 to better demonstrate the lack of relationship between M23-Ab and M1-Ab titres.

transfected or untransfected HEK293 cells, before being incubated with M23-transfected HEK cells, which were used for FACS. Pre-adsorption with untransfected HEK cells did not reduce FACS scores whilst pre-adsorption with M23-transfected cells markedly reduced FACS scores to a similar degree for all samples (Figure 4.7). Pre-adsorption with M1-transfected cells reduced FACS scores only slightly in the sample from patient NMO 007 (no detectable M1 antibodies; Figure 4.7A), to a comparable degree as pre-adsorption with M23 cells in the sample from patient NMO 145 (equal M1 and M23 binding; Figure 4.7B) and by around 50% in the sample from patient NMO 004 (2:1 M23:M1 binding; Figure 4.7C).

4.3.6 M23 and M1 antibody titres and disease activity

4.3.6.1 M23 and M1 antibody titres at disease onset

Onset attack samples were available from five patients (four patients with LETM onset attacks and one with an ON onset attack), all of whom had detectable antibodies to both isoforms. The median M23-Ab titre at presentation was 2560 (range 1920 to 20480) and the median M1-Ab titre at presentation was 960 (range 60 to 5120). The M23:M1 ratio at presentation ranged from 32:1 to 1:1. Onset attack severity (measured by nadir EDSS score) did not correlate with either M23-Ab or M1-Ab titres.

4.3.6.2 M23 and M1 antibody titres in relapse and remission

Twenty six relapse samples were available from 17 patients (range 1 to 6 relapses per patient). All patients had detectable M23-Ab during relapse but the titres at which relapses occurred varied markedly (median 2560; range 40 to 81920). M1-Ab titres at relapse also varied markedly across patients (median 180; range 0 to 20480). Two patients, however, had undetectable M1-Ab during relapses; one of these had undetectable M1-Ab in 6 of 6 relapse samples with M23-Ab of 320 to 2560. This patient had samples available from LETM, ON and brain relapses and M1-Ab were absent in all.

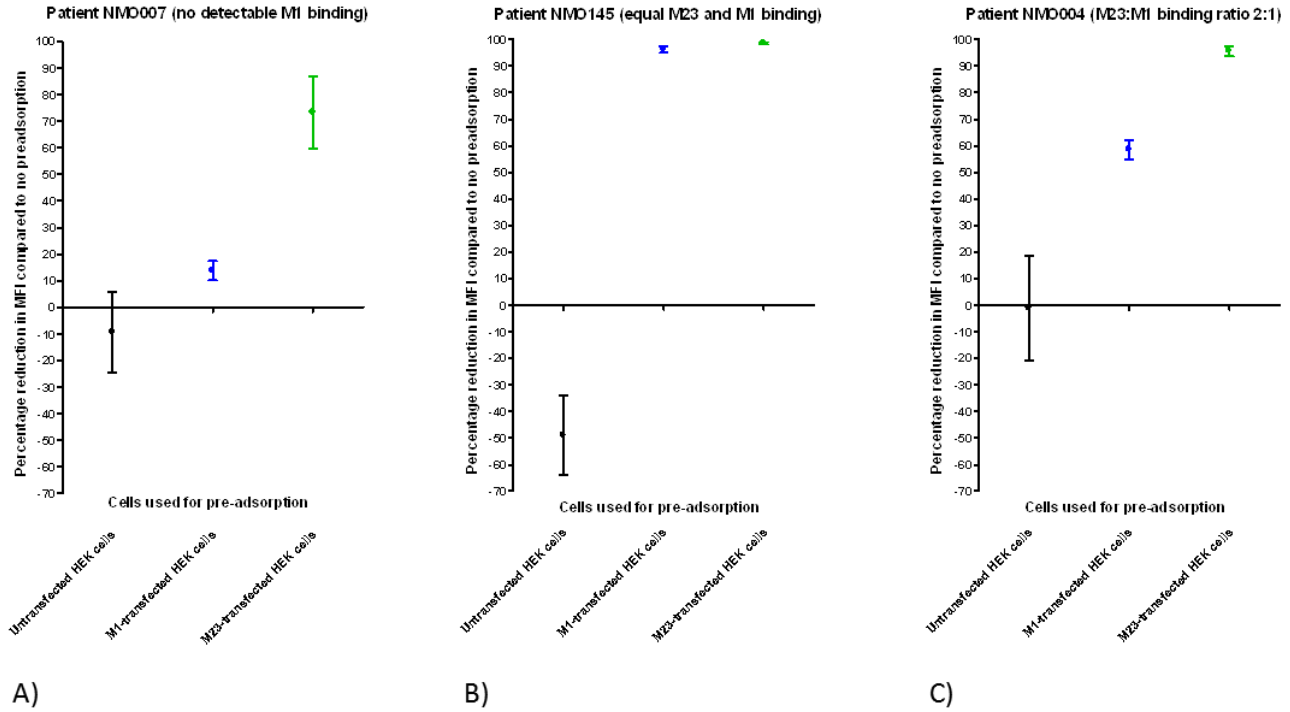


Figure 4.7. Effect of pre-adsorption on FACS scores. Sera from patients with no M1 antibody binding (A), equal M23 and M1 binding (B) and M23:M1 binding ratio of 2:1 (C) were pre-adsorbed on untransfected (black), M1-transfected (blue) or M23-transfected (green) HEK cells, before being incubated with M23-transfected cells and analysed on FACS. Pre-adsorption on untransfected HEK cells did not reduce FACS scores, whilst pre-adsorption with M23-transfected cells reduced FACS scores by at least 75% for all samples. Pre-adsorption on M1 cells reduced FACS scores to a comparable degree as pre-adsorption with M23 cells in the sample with equal M23 and M1 binding (B), barely reduced FACS scores in the sample with no detectable M1 binding (A), and reduced FACS scores by approximately 50% in the sample with an M23:M1 binding ratio of 2:1 (C). Plots represent mean of three experiments.

Group analysis showed that median M23-Ab titres were significantly higher during relapse than during remission (Figure 4.8; p=0.016; Mann-Whitney test). Median M1-Ab titres were also higher during relapse than in remission but this did not reach statistical significance (Figure 4.8; p=0.182).

Twelve patients had samples available from periods of both relapse and remission. Five of these patients had M23-Ab titres higher during a period of remission than during relapse and the same was true for M1-Ab titres in three patients. On the other hand, patients could have extremely high M23-Ab and M1-Ab titres without experiencing a relapse (up to 81920 and 15360 respectively). Thus, titres did not correlate well with disease activity. Paired samples from relapses and following remission periods were available in 11 patients (14 samples). Neither M23-Ab nor M1-Ab titres were significantly higher during relapse than during remission in these paired samples (Figure 4.9; p=0.191 for M23; p=0.577 for M1; Wilcoxon matched pairs test).

Examples of serial antibody determinations are shown in Figure 4.10. Although some relapses were associated with a rise in antibody titres (Figure 4.10A), definite relapses (MRI proven, new clinical signs) could occur without a rise in antibody titres (Figure 4.10B). For one patient, 11 samples were available (6 relapse, 5 remission), allowing inter-patient comparison of relapse and remission titres. M1-Ab were undetectable in all samples, as mentioned above, and there was no significant difference between median relapse and remission M23-Ab titres (1600 vs. 1280; p=0.792).

It was also noted that, over time, relapses tended to occur at lower antibody titres (Figure 4.10B). Five patients had serum samples available for more than one relapse. In four of these five patients, M23 and M1 antibody titres were highest in the earliest relapse.

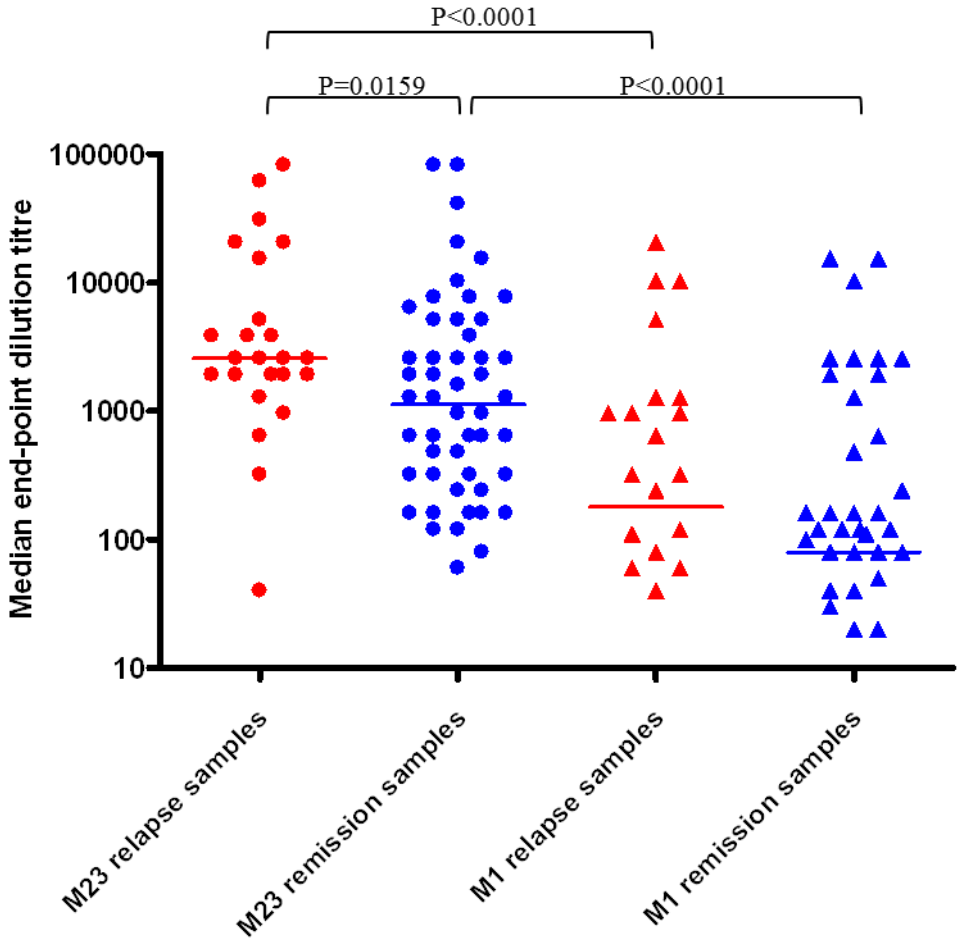


Figure 4.8. Median M23 and M1 antibody titres during relapse and remission. Group analysis showed that median M23-Ab titres were significantly higher during relapse than during remission [median 2560 (range 40 to 81920) vs. 1120 (<20 to 81920); p=0.0159]. Median M1-Ab titres were also higher during relapse than in remission but this did not reach significance (180 (<20 to 20480) vs. 80 (<20 to 15360); p=0.1822). Median M23-Ab titres were significantly higher than M1-Ab titres during both relapse (p<0.0001) and remission (p<0.0001). Note log10 scale on y axis.

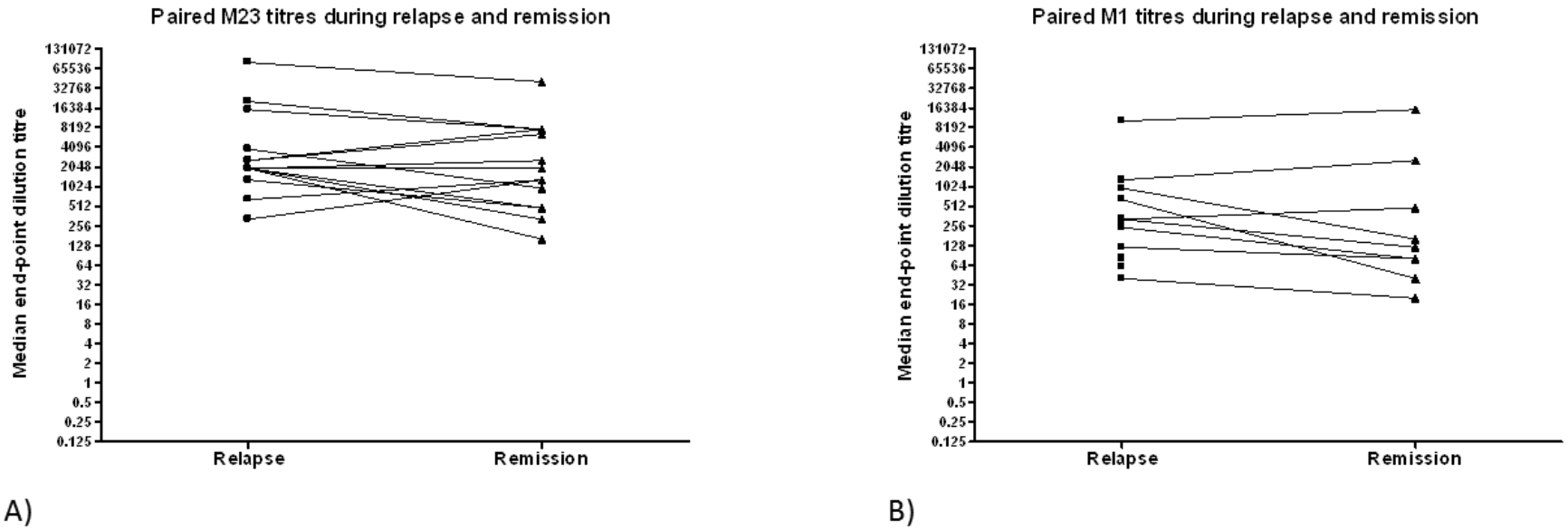


Figure 4.9. Paired M23 and M1 antibody titres during relapse and remission. A) Median M23-Ab titres during relapse were not significantly different from M23-Ab titres in a following remission sample [median 1920 (range 320 to 81920) vs. 1600 (160 to 40960); p=0.191; Wilcoxon's matched pairs]. B) Median M1-Ab titres were also no different between paired relapse and remission samples [240 (<20 to 10240) vs. 80 (<20 to 15360); p=0.577]. Note log2 scale on y axes.

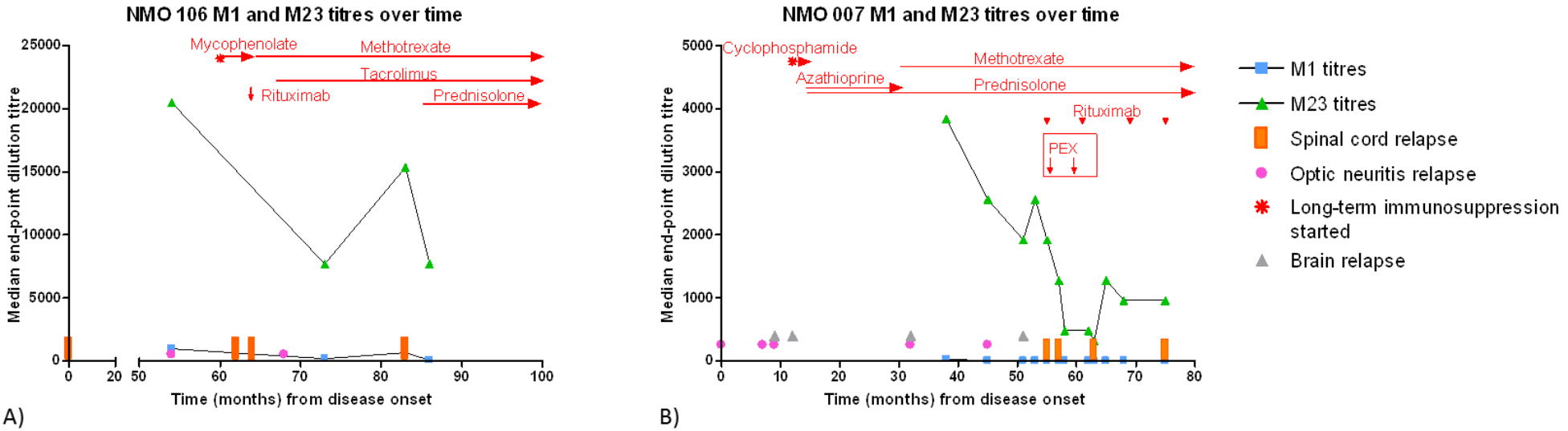


Figure 4.10. Relationship between antibody titres and relapses. A) Shows a rise in both M23 and M1 antibody titres in association with an LETM attack at month 83. B) Shows multiple relapses in a patient without any increase in antibody titres. No antibodies to M1 could be detected in any samples from this patient.

4.3.6.3 M23 and M1 antibody titres in different types of relapse

The 26 relapse samples were taken largely from LETM (n=18) or ON (n=5) attacks; only one sample was taken from an attack of simultaneous LETM/ON and two samples were taken from attacks affecting the brain and/or brainstem. There were no significant differences in median M23-Ab or M1-Ab titres between the different types of attacks, though of note M1-Ab were undetectable in both brain attacks (Figure 4.11). Relapse severity (measured by nadir EDSS score) did not correlate with either M23 or M1 antibody titres.

4.3.7 M1 and M23 antibody titres over time

Samples were measured longitudinally in 15 patients (range 2 to 11 samples per patient). Over time, changes in M23 and M1 antibody titres within a patient tended to occur in parallel (Figure 4.12). M1-Ab became undetectable in four patients after treatment whilst M23-Ab were still detected in all but one but at lower levels.

4.3.7.1 M23 and M1 antibodies can be present years before the onset of disease.

Five archived serum samples from one NMOSD patient (NMO080), with a previous history of typical early-onset MG, were available from up to 15 years prior to NMO onset. Whilst neither M23 nor M1 antibodies were detectable 124 months prior to NMO onset, M23-Ab became detectable 90 months, and M1-Ab 87 months, before the first NMO attack. Titres for both isoforms then increased between this time and development of the onset attack (Figure 4.12D).

4.3.7.2 Effect of treatment on M23 and M1 antibody titres

Group analysis revealed that there were no significant differences in median M23-Ab titres or median M1-Ab titres between samples taken on treatment (n=58) compared to samples taken off treatment (n=20) (Figure 4.13A). However, within an individual patient, antibody titres seemed to decrease following initiation of treatment (Figure 4.12E-F). However, this may be confounded by the fact that treatment was started during, or shortly after relapse, whereas follow-up samples were taken during remission. Paired pre and post-treatment M23 and M1 antibody titres were available

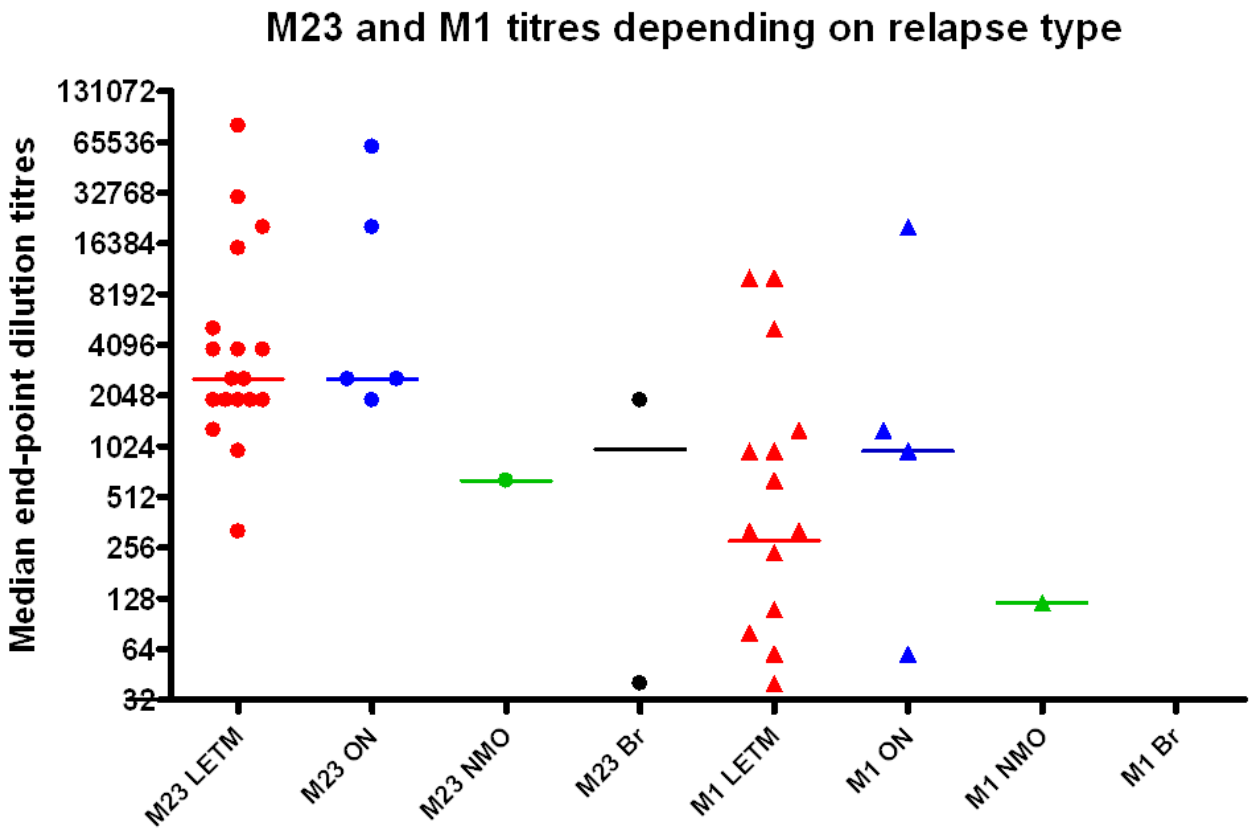


Figure 4.11. M23 and M1 antibody titres in different types of relapse. Kruskal-Wallis analysis revealed no significant differences in either median M23-Ab titres or M1-Ab titres between attack types (p=0.128 and p=0.290 respectively). Note log2 scale on y axis. LETM = longitudinally extensive transverse myelitis; ON = optic neuritis; NMO = simultaneous optic neuritis and myelitis; Br = brain or brainstem relapse.

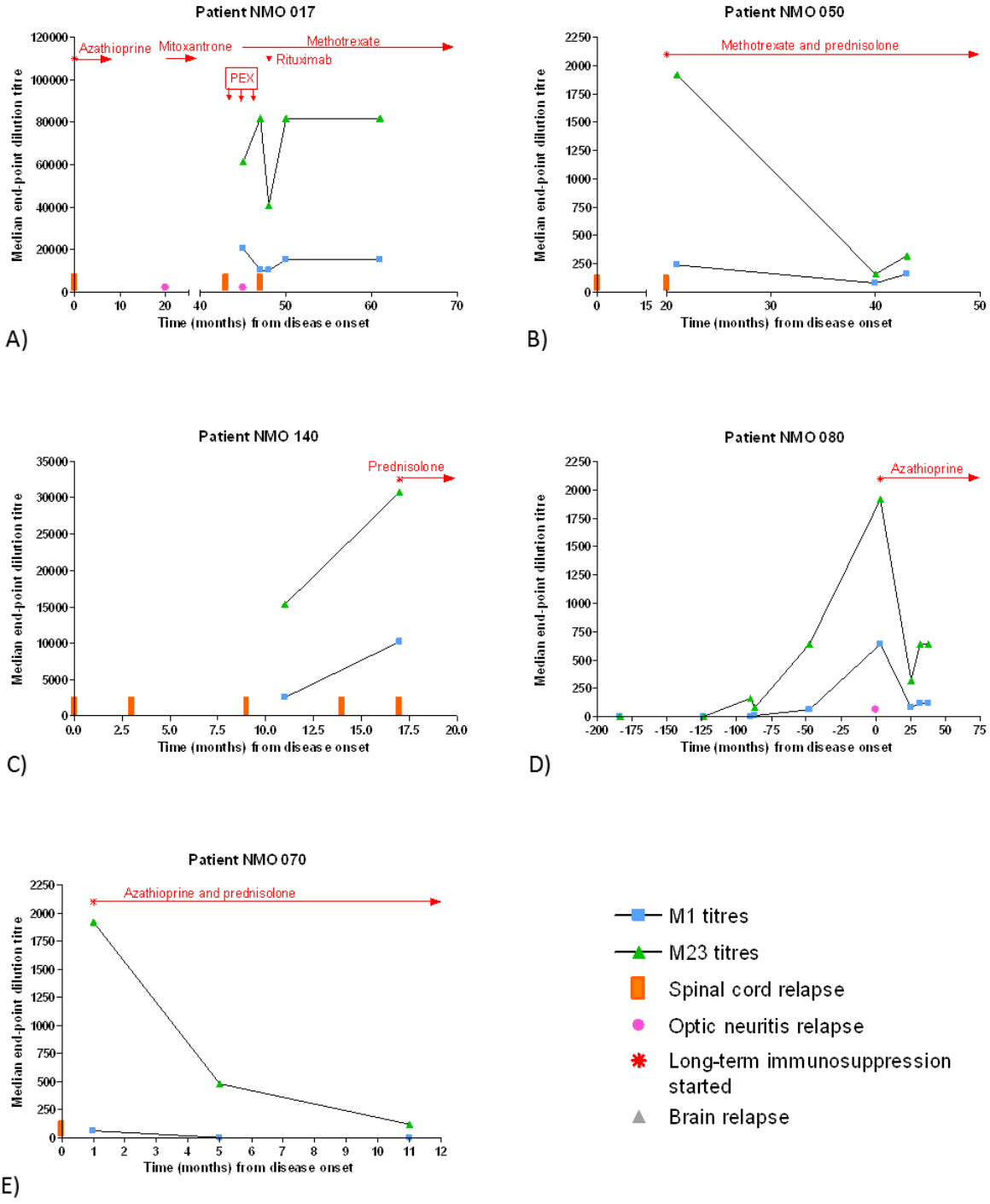


Figure 4.12. Examples of longitudinal M1 and M23 antibody titres over time. In general, changes in M23 and M1 antibody titres tended to occur in parallel (A-E). D) Archived sera available in one patient revealed that AQP4-Ab appeared in the sera around seven years prior to disease onset. Within individual patients, treatment seemed to lead to a reduction in antibody levels (B, D, E).

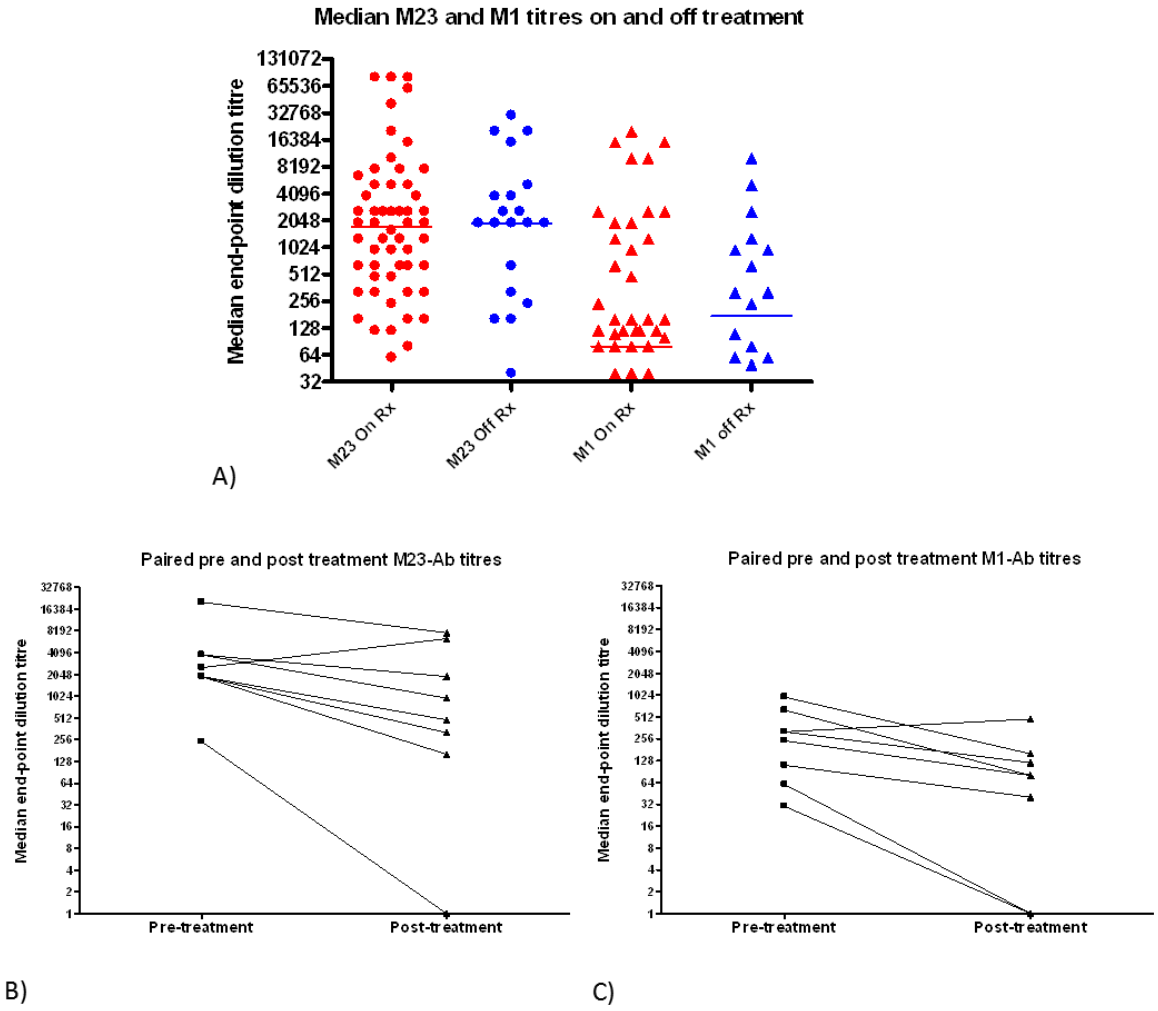


Figure 4.13. Treatment effects on M23 and M1 antibody titres. A) Group analysis showed that there was no significant difference in median on or off treatment titres for either M23 or M1 (median 1760 vs. 1920, p=0.591 for M23; 80 vs. 175, p=0.255 for M1). Although both M23 and M1 antibody titres decreased in seven of eight patients with paired samples available, there was no significant difference in either M23 (B) or M1 (C) antibody titres between paired pre and post-treatment samples (median 2240 vs. 720, p=0.148 for M23; median 280 vs. 80, p=0.055 for M1). Note log2 scale on y axes.

in 8 patients, and although in 7 of these both M23 and M1 antibody titres decreased following initiation of treatment, the reductions were not statistically significant (Figure 4.13B).

One patient (NMO037) stopped treatment after 89 months of disease and has remained relapse free off treatment for seven and a half years. Her M23-Ab titres, 81 months after treatment cessation, are only one third of what they were at initiation of treatment, and M1-Ab titres are undetectable.

4.3.8 Influence of relative M23 and M1 antibody titres on phenotype

4.3.8.1 M23 and M1 antibody titres and phenotypic variations

It was noted in chapter 3 that some patients have limited disease phenotypes. To investigate whether this may be related to isoform specificity differences, samples from patients with relapsing ON (≥ 3 attacks of ON without involvement of other CNS regions; n=6) were compared with samples from patients with relapsing LETM (≥ 3 attacks of LETM without involvement of other CNS regions; n=8) and with samples from patients with NMO (attacks of both ON and LETM; n=8). M23 and M1 antibody titres were measured on the earliest available sample from each patient. The median time from disease onset to date of first sample did not differ between the groups (p=0.148). There were no significant differences between median M23 or M1 antibody titres in the three phenotype groups (Figure 4.14).

4.3.8.2 M23 and M1 antibody titres at extremes of age

Because of the age at onset differences in clinical phenotype and disability noted in chapter 3, potential isoform specificity differences at extremes of age were investigated. M1 and M23 antibody titres were measured on the earliest available sample for patients with age at onset <16 years (n=5) or age at onset >70 years (n=4). The median time from disease onset to date of first sample did not differ between the groups (p=0.111). There were no significant differences between median M23 or M1 antibody titres between the two groups (Figure 4.15).

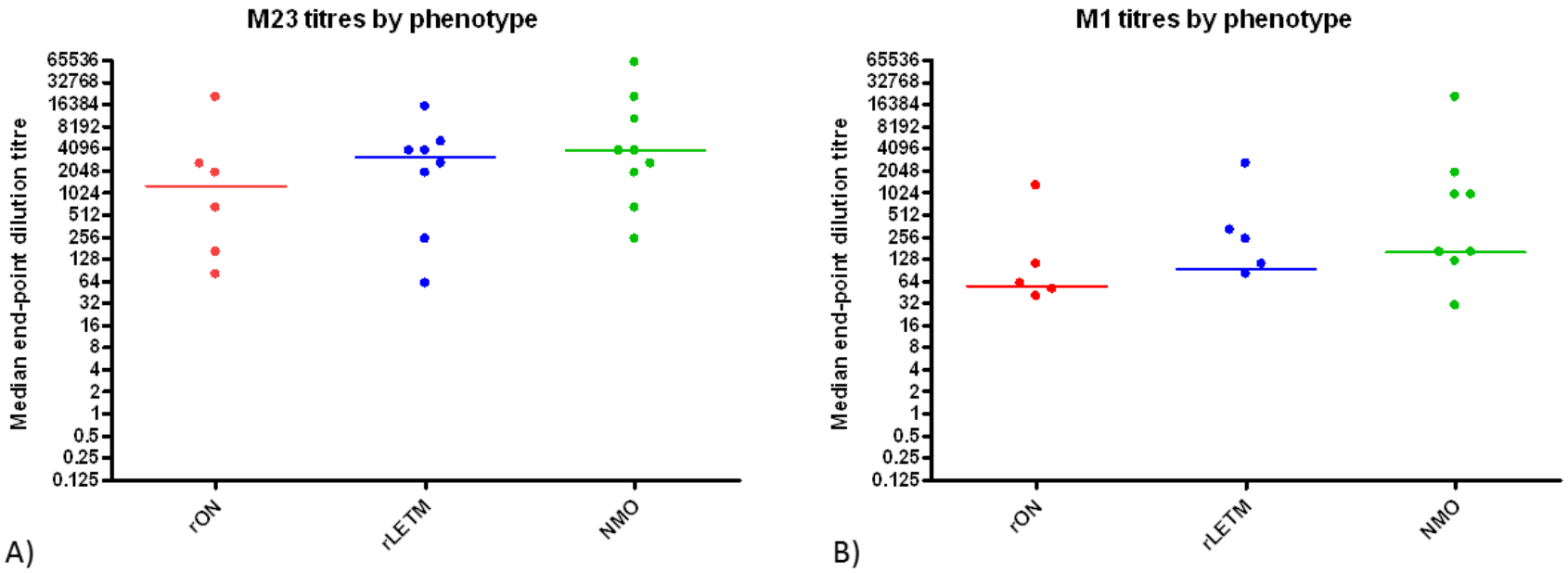


Figure 4.14. M23 and M1 antibody titres in different phenotypic groups. M23 and M1 antibody titres were measured on the earliest available sample from patients with relapsing ON (≥ 3 attacks of ON without LETM, n=6), relapsing LETM (≥ 3 attacks of LETM without ON, n=8) and those with the full NMO phenotype (≥ 1 attack of ON and ≥ 1 attack of LETM, n=8). A) Median M23-Ab titres did not differ significantly between patients with a relapsing ON phenotype [median 1280 (range 80 to 20480)], those with relapsing LETM [3200 (60 to 15360)] and those with the full NMO phenotype [3840 (240 to 61440); p=0.364, Kruskal-Wallace test]. B) Median M1-Ab titres also did not differ between the groups [median 55 (range <20 to 1280) for relapsing ON; 95 (<20 to 2560) for relapsing LETM; 160 (<20 to 20480) for the full NMO phenotype; p=0.852]. Note log2 scale on y axes. rON = relapsing ON; rLETM = relapsing LETM, NMO = full NMO phenotype.

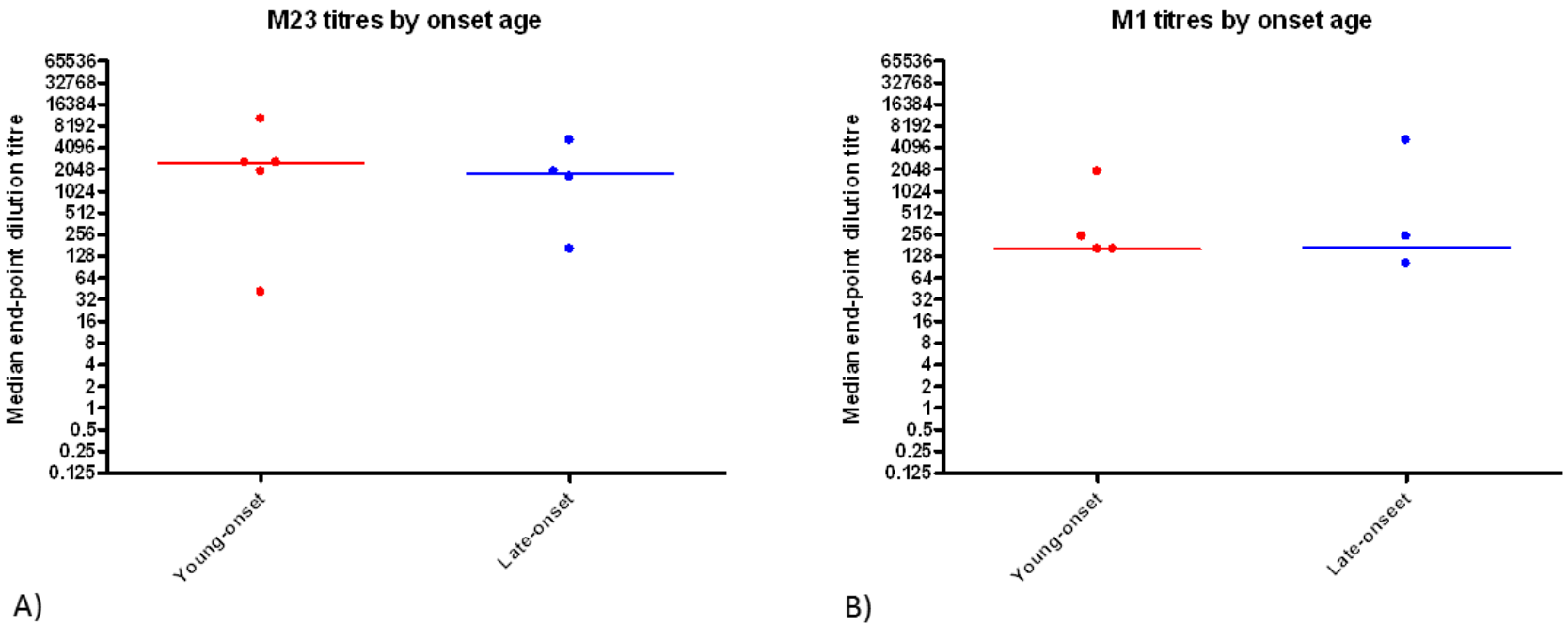


Figure 4.15. Onset age and M23 and M1 antibody titres. M23 and M1 antibody titres were measured in the earliest available sample from young-onset (age <16 years at onset, n=5) and late-onset (age >70 years at onset, n=4) patients. A) The median M23-Ab titre in young-onset patients [median 2560 (range 40 to 10240)] did not differ significantly from the median M23-Ab titre in late-onset patients [1760 (160 to 5120); p=0.556, Mann-Whitney test]. B) The median M1-Ab titre in young-onset patients [160 (<20 to 1920)] also did not differ significantly from the median M1-Ab titre in late-onset patients [170 (<20 to 5120); p=1.000]. Note log2 scale on y axes.

4.3.8.3 M23 and M1 antibody titres and ethnicity

Isoform specificities were compared between samples from patients of different ethnicities (Caucasian n=25; Asian n=2; Afro-Caribbean n=7). The earliest available sample from each patient was used and the median time from disease onset to date of first sample did not differ between the groups (p=0.156). The median M23 and M1 antibody titres did not differ significantly between the three ethnic groups (Figure 4.16).

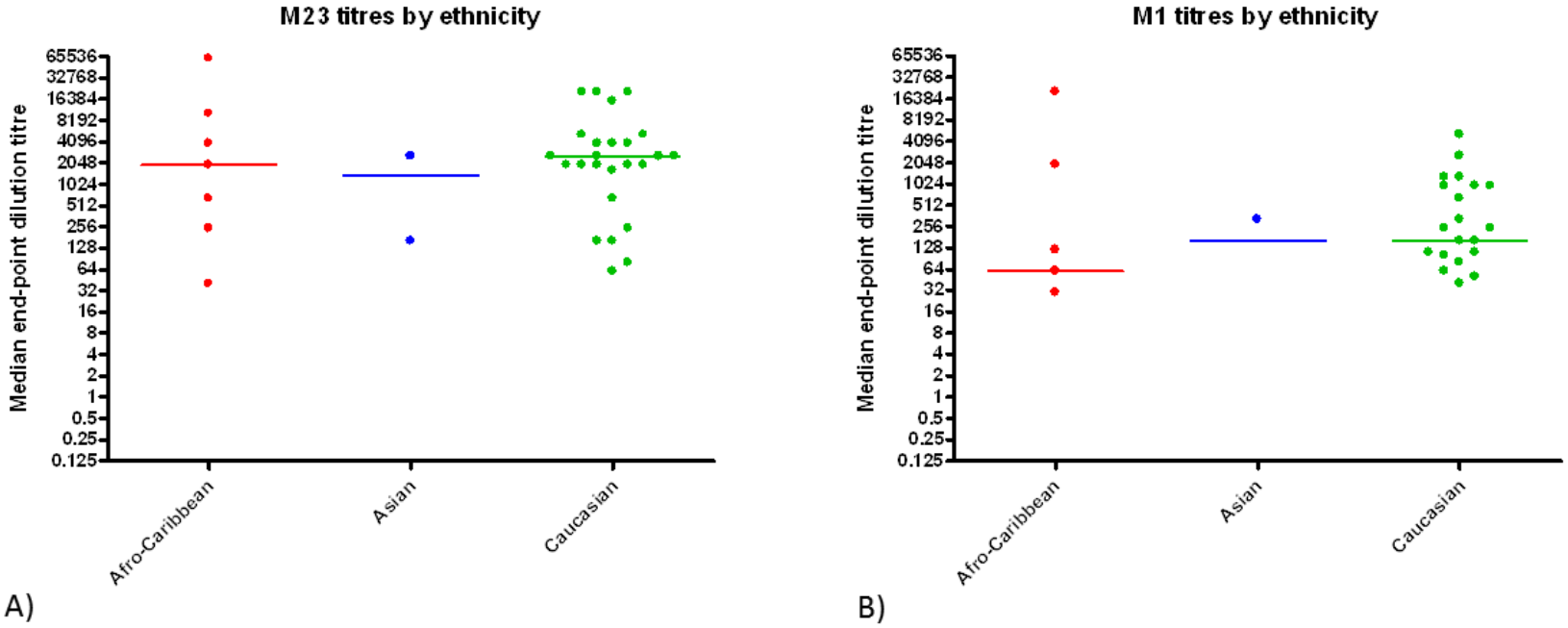


Figure 4.16. M23 and M1 antibody titres in different ethnic groups. M23 and M1 antibody titres were measured on the earliest available sample from patients of Afro-Caribbean (n=7), Asian (n=2) and Caucasian (n=25) ethnicities. A) Median M23-Ab titres did not differ significantly between patients of Afro-Caribbean, Asian and Caucasian ethnicity [median 1920 (range 40 to 61440) vs. 1360 (160 to 2560) vs. 2560 (60 to 20480); p=0.747; Kruskal-Wallace test]. B) Median M1-Ab titres also did not differ significantly between the three ethnic groups [60 (<20 to 20480) vs. 160 (<20 to 320) vs. 160 (<20 to 5120); p=0.218]. Note log2 scale on y axis.

4.4 Discussion

Although there have been previous studies demonstrating that patients' sera react differently with the two AQP4 isoforms, none have comprehensively studied unselected patients at different stages of disease in a systematic and quantitative manner. We have confirmed previous findings (Mader, Lutterotti et al, 2010; Crane, Lam et al, 2011) that relative AQP4-Ab binding to M23 and M1 isoforms differs between patients and have investigated the potential clinical significance of this differential binding. We could not identify an association between these differences and any clinical characteristics of disease such as age at onset, clinical phenotype, ethnicity and disease severity.

In concordance with other studies (Nicchia, Mastrototaro et al, 2009; Mader, Lutterotti et al, 2010; Crane, Lam et al, 2011; Iorio, Fryer et al, 2012) we found that antibody binding to the M23 AQP4 isoform is stronger than binding to the M1 AQP4 isoform in the majority of patients. Thus, assays employing the M23 isoform should be used in diagnostic laboratories. Whilst 97% of samples in our cohort, which were taken in both relapse and remission phases of disease, were positive for M23-Ab, only 67% were positive for M1-Ab and no samples were positive only for M1-Ab. Although M1-Ab were generally only undetectable when M23-Ab were present at relatively low levels, no threshold cut-off could be identified and the relative binding to the two isoforms was not predicted by end-point dilutions of either isoform. Additionally, there were individuals with equal binding affinities and others with relatively high M23-Ab titres but undetectable M1-Ab and overall, the ratio of M23 to M1 binding varied markedly across patients. These findings suggest that the reason for differences in isoform binding is not simply due to variability in cell surface expression of the two isoforms. One possibility is that AQP4-Ab bind better to M23 because it is expressed in OAPs, which could enable divalent binding to take place on the cell surface, which would be much less likely than with the less clustered M1 isoform. An analogous situation in MG has been demonstrated where clustering of the acetylcholine receptor on the surface of HEK cells identified acetylcholine receptor antibodies in patients who were negative for binding to unclustered acetylcholine receptor antibodies in solution (Leite, Jacob et al, 2008). However, it has

previously been shown that purified AQP4-Ab Fab fragments, which can only bind monovalently, have comparable affinity to intact AQP4-Ab, suggesting that it is not divalent binding due to clustering of M23 that accounts for enhanced reactivity to the M23 isoform (Crane, Lam et al, 2011). There is evidence that higher order structures such as OAPs present epitopes that are not found on M1 expressing cells, perhaps because there are conformational changes when M23 forms these clusters (Phuan, Ratelade et al, 2012). It is unclear, even if AQP4-Ab have preferential binding for M23 in OAPs over M1 heterotetramers, why the relative binding to M1 varied across patients. Serum samples with equal M23 end-point dilution titres from three patients had very different M1 end-point dilutions and pre-adsorption on M1-expressing cells reduced M23 binding to a strikingly different degree between samples. Pre-adsorption on M1-expressing cells did not reduce M23 binding in the sample with no detectable M1 antibodies, whilst it led to almost complete loss of M23 binding in a sample with equal M23:M1 binding, confirming that the individual samples had differential M1 binding despite comparable M23 binding. This may be accounted for by different specificities of individual monoclonal antibodies within patients. However, relative M23:M1 binding within a patient remained fairly constant over time, whereas it may be expected that monoclonal antibody production varies depending on factors such as disease activity and disease duration.

We recently found (chapter 3; Kitley, Leite et al, 2012) that clinical features and disease course in NMO are influenced by age at disease onset and ethnicity. Young patients had a propensity to ON attacks with consequent visual disability, whilst older patients more often presented with myelitis and were more at risk of motor disability. Ethnic differences were also noted, with Afro-Carribean patients having a greater propensity for optic nerve involvement. Thus, we investigated whether differential binding to the two AQP4-Ab isoforms could in part explain these differences. However, although there was clear variation in relative M23:M1 binding across patients, we could not find any relationship between these variations and patient factors. We and others (Nagaishi, Takagi et al, 2011) have also noted different phenotypic categories, with a proportion of patients having striking anatomically limited forms of disease and we looked into whether this may be

related to relative isoform binding specificities. However, there were no differences in relative binding specificities between patients with typical NMO and those with more limited phenotypes. What's more, when sera from all relapses were compared, neither M23 nor M1 antibody titres were significantly different between different types of attacks, and one patient with samples available from LETM, ON and brain relapses had absent M1-Ab in all types of attack, suggesting that it is not isoform binding specificities that dictate anatomical site of attack.

We found that antibodies against both isoforms were present in all available onset attack samples, which contradicts an earlier report that M1-Ab only appear with longer disease duration (Mader, Luterotti et al, 2010). This may partly reflect the sensitivity of our assays, but we did also find that M1-Ab tended to disappear over time following initiation of immunosuppression in some patients. Although group analysis in the current study showed no difference in M23-Ab or M1-Ab titres between on and off treatment samples, within individual patients, titres decreased following initiation of treatment in the majority of patients. It is difficult to be sure that this reduction was specifically treatment driven or whether a reduction in antibody titres may have occurred naturally over time. One patient had treatment withdrawn and has remained relapse free off treatment for seven and a half years and antibody levels have fallen successively, suggesting that antibody levels can fall naturally during periods of disease quiescence. However, this is an exceptional case and unfortunately no other serial samples were available in untreated patients. Previous studies have found that within individual patients, treatment is associated with a significant reduction in AQP4-Ab titres (Jarius, Aboul-Enein et al, 2008; Kim, Kim, et al, 2011b).

As mentioned in chapter 1, there is conflicting evidence as to whether AQP4-Ab titres across NMO patients are of clinical or prognostic significance, with some studies suggesting that higher titres may be associated with longer spinal cord lesions (Takahashi, Fujihara et al, 2007), greater likelihood of visual disability (Takahashi, Fujihara et al, 2007) and a greater chance of patients having the 'full' NMO phenotype (Takahashi, Fujihara et al, 2007; Matiello, Lennon et al, 2008; Mader, Lutterotti et al, 2010) whilst others have shown no correlation between titres and lesion length (Waters, Jarius et al, 2008) or relapse severity (Hayakawa, Mori et al, 2008; Hinson,

McKeon et al, 2009). Also, some studies have shown that AQP4-Ab titres are higher during relapse than in remission (Takahashi, Fujihara et al, 2007; Waters, Jarius et al 2008; Jarius, Aboul-Enein et al 2008; Jarius, Franciotta et al, 2010), whilst others have not (Hayakawa, Mori et al, 2008; Hinson, McKeon et al, 2009; Isobe, Yonekawa et al, 2013). One problem is that analyses are usually performed across groups, whereas it is apparent that AQP4-Ab titres, both in relapse and remission, vary markedly across individuals. Thus, group analysis may hide any differences. Studies that have looked within individuals have found that, in general, relapses are usually associated with a rise in titres, but can occur without (Kim, Kim, et al, 2011; Kim, Lee et al, 2012; Isobe, Yonekawa et al, 2013). These studies all used ELISA scores at a single serum dilution to compare titres and did not provide a definition of relapses, which can be difficult to determine with certainty. Here, we have confirmed using different methodology, and employing a strict definition of relapses, that radiologically-proven relapses can occur without a rise in AQP4-Ab titres. Titres at which relapses occurred varied markedly both between and within individuals and we could not identify a threshold level above which relapses occurred. Thus, whilst measurement of AQP4-Ab titres within an individual may be helpful in assessing response to treatment, with rising titres possibly indicating a need for treatment escalation, they cannot predict with certainty whether imminent disease activity is likely to occur. It was also noted that, over time, relapses tended to occur at lower antibody titres, which may suggest that the threshold value for relapse decreases over time. This may be because the blood-brain barrier has already been breached and thus fewer antibodies are needed to initiate CNS inflammation, or that the potency of the antibodies somehow increases over time, or that following a first attack, the CNS is sensitised to further attacks. Alternatively it may be that T cells and other inflammatory mediators are more involved in subsequent attacks, following their priming during initial attacks. We also found that M23 and M1 AQP4-Ab were present several years prior to disease onset in one patient for whom archived samples were available. Taken together, these findings suggest that the pathogenesis of NMO and the occurrence of relapses must be related to factors other than just AQP4-Ab.

It was recently shown that binding to M23 but not M1 activates complement on cell-surface membranes resulting in cell death (Hinson, Romero et al, 2012; Phuan, Ratelade et al, 2012) and it had previously been shown that the degree of complement activation by patient sera correlated with attack severity (Hinson, McKeon et al, 2009). Therefore, one may expect patients with higher M23-Ab titres to have more severe attacks. Alternatively, high M1-Ab titres could inhibit disruption of OAP formation by M1 AQP4, resulting in larger OAPs with more complement activating ability. However, we found no correlation between M23-Ab or M1-Ab titres and attack severity, either at disease onset or in subsequent relapses.

This work has several limitations. Firstly, many subgroup analyses involved small numbers of patients and thus we cannot discount the fact that statistical analyses were underpowered to detect any differences. Secondly, all sera came from patients seen within a specialist NMO referral centre and thus there may be an element of referral bias. Thirdly, some data were collated retrospectively, which could introduce recall bias with respect to timings of relapses and treatment. However, the majority of patients were inpatients at the time of relapses and thus detailed clinical information was available from case notes.

In summary, whilst we have shown that the relative binding to the M23 and M1 AQP4 isoforms varies between patients, we could not identify an association between these differences and any clinical characteristics of disease.

Chapter 5: Clinical characteristics and diagnoses of seronegative NMO/NMOSD patients

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5.1 Introduction

Until the discovery of AQP4-Ab, NMO was defined clinically as the co-existence of inflammatory myelitis and ON without symptomatic disease outside of these regions. However, the high specificity of AQP4-Ab has enabled broadening of the clinical phenotype of NMO. In chapter 3 we showed that 47% of AQP4-Ab positive patients had limited or atypical forms of disease, most commonly monophasic or recurrent LETM. The NMO diagnostic criteria do not currently include such patients but most specialists agree that they should be treated the same as those with the full NMO phenotype, with early and aggressive immunosuppression. The diagnostic criteria need updating to reflect the expanding spectrum of NMO with AQP4-Ab. On the other hand, it is less clear how to classify and treat patients with NMO/NMOSD without AQP4-Ab. This is important for the correct definition of patients in clinical studies, which will influence outcome predictions and patient management.

LETM is a hallmark of NMO but is not specific to NMO/NMOSD with AQP4-Ab. Other causes include non-AQP4-Ab mediated inflammatory disorders, infection, malignancy and paraneoplastic disease, metabolic and vascular aetiologies (Kitley, Leite et al, 2012b). Results of investigations and the subsequent clinical picture that eventually enable a diagnosis to be made are often not available in the acute setting. Thus, exploring clinical and radiological features at presentation that can help suggest one diagnosis over another is useful. In this chapter we describe the 'spectrum' of diseases causing LETM that are not due to AQP4-Ab disease and identify useful features in the acute setting that can help guide diagnosis.

5.1.1 Aims

The main aim of this chapter was to clinically characterise NMO/NMOSD patients without AQP4-Ab and to determine whether there are clinical and paraclinical features in the acute setting that can help distinguish these patients from those with AQP4-Ab. The specific aims were:

- To define the clinical and paraclinical characteristics of seronegative NMO/NMOSD patients
- To compare these characteristics with those of patients who are AQP4-Ab positive
- To describe alternative diagnoses in seronegative NMO/NMOSD patients

5.2 Methods

The Oxford NMO clinical database was searched for patients with a history of LETM, either at disease presentation or later on in the disease course, seen within the clinical service between 2008 and May 2012. All adult patients with a history of LETM, including those with previous attacks of CNS inflammation were included; patients <16 years at LETM were excluded. For patients with multiple LETM attacks, only features of the index LETM were analysed.

Demographic, clinical and paraclinical data were collated retrospectively from the NMO clinical database and from case notes. The European Database System for Multiple Sclerosis (EDMUS; Confavreux, Compston et al, 1992) scoring system was used to estimate disability during and after the attack. EDMUS scores were used in preference to EDSS scores because they can be more accurately assigned to retrospective data. MRI features were reviewed by a Neuroradiologist with an interest in inflammatory disorders. All patients were tested for AQP4-Ab at least twice on the Oxford diagnostic CBA, and by FACS. There was complete agreement in the positivity and negativity of all patients. MOG-Ab were tested in all patients using a CBA.

As this was a retrospective study, no routine panel of investigations was performed for every patient but clinical diagnoses were based on imaging (brain and spinal cord MRI), CSF examination in most, and infection, autoimmune, paraneoplastic and metabolic screening.

Statistical analysis was performed using GraphPad Prism v4. Mann-Whitney U test was used to compare continuous variables and Fisher's exact test was used to compare frequencies. Statistical significance was set at $p<0.05$.

5.3 Results

Seventy six patients were included. Forty four of these (58%) tested positive for AQP4-Ab and 32 (42%) were AQP4-Ab negative. The median follow up time since LETM was significantly longer in the AQP4-Ab positive group [61.35 months (range 2.3 to 260.2)] than the AQP4-Ab negative group [25.04 months (range 1.9 to 169.4); p=0.010].

5.3.1 Demographic differences between AQP4-Ab positive and negative cases

There were differences between AQP4-Ab positive and negative cases (Table 5.1). AQP4-Ab positive patients were older at LETM and comprised a greater proportion of females (Figure 5.1). A higher frequency of co-existing autoimmune disorders was evident in the AQP4-Ab positive cases. Thirteen patients (12 AQP4-Ab positive) had a coexisting autoimmune disease. These included coeliac disease (n=1), pernicious anaemia (n=1; patient additionally had Sjögren's syndrome), thrombotic thrombocytopaenic purpura (n=1), Sjögren's syndrome (n=3), SLE (n=1), thyroid disease (n=3) and MG (n=3). The only seronegative patient with an autoimmune disorder had vitiligo; this patient had relapsing ON and LETM.

5.3.2 Onset attack features in AQP4-Ab positive and negative cases

Differences in features of the onset attack are shown in Table 5.1. There was a trend towards more patients having had previous ON in the AQP4-Ab positive group. By contrast, more patients experienced simultaneous or rapidly sequential ON with their LETM as part of a 'classic' Devic's syndrome [as originally described (Devic, 1894)] in the AQP4-Ab negative group. There were no differences in the proportions of patients with a preceding infection or preceding pain between the groups although there was a trend towards preceding vomiting being more common in the AQP4-Ab positive patients. The first neurological symptom of TM was similar in both groups (sensory

Table 5.1. Demographic, clinical and paraclinical features of AQP4-Ab positive and negative patients

Clinical/Paraclinical Feature	AQP4-Ab positive (n=44)	AQP4-Ab negative (n=32)	P value
% females	86	44	<0.0001
Mean age at LETM ± s.d.	45.3 ± 14.8	37.7 ± 16.1	0.02
Caucasian ethnicity [n(%)]	32 (73%)	24 (75%)	ns
Co-existing autoimmune disorder [n(%)]	12 (27%)	1 (3%)	<0.01
Past history of ON [n(%)]	12 (27%)	5 (16%)	ns
Devic's syndrome (ON+TM) at onset [n(%)]	2 (5%)	8 (25%)	0.01
Preceding vomiting [n(%)]	10 (of 35) (29%)	3 (of 31) (10%)	0.05
First neurological symptom	N=38	N=31	
Back/neck pain	15 (39%)	6 (19%)	ns
Numbness/paraesthesia	13 (34%)	10 (32%)	ns
Limb weakness	7 (18%)	5 (16%)	ns
Urinary retention	0 (0%)	5 (16%)	0.02
Other	3 (7%)	5 (17%)	ns
Median nadir EDMUS score (range)	7.5 (0 to 9)	8.0 (3 to 8)	ns
Median EDMUS score at recovery (range)*	2.0 (0 to 7)	3.0 (0 to 7)	ns
Proportion left motor disabled (EDMUS ≥ 6)	36%	35%	ns
Proportion left wheelchair dependent	5%	6%	ns
MRI spine	N=33	N=29	
All regions of cord involved	1 (3%)	5 (17%)	ns
Involvement of conus	2 (6%)	10 (34%)	<0.01
Mean length of longest lesion ± s.d.	7.48 ± 3.39	7.66 ± 5.12	ns
MRI brain	N=32	N=29	
Normal	12 (38%)	11 (38%)	ns
Non-specific white matter lesions	11 (34%)	17 (59%)	ns
MS-like	4 (13%)	3 (14%)	ns
CSF	N=19	N=25	
Marked CSF pleocytosis >50/mm^3	3 (16%)	11 (44%)	0.06
Raised CSF protein >1g/L	3 (16%)	9 (35%)	ns
CSF oligoclonal bands	3 (16%)	0 (0%)	0.07

*Only patients with follow up >6 months were included in the analysis. LETM = longitudinally extensive transverse myelitis; s.d. = standard deviation; n = number; ON = optic neuritis; TM = transverse myelitis; EDMUS = European Database System for Multiple Sclerosis; MRI = magnetic resonance imaging; MS = multiple sclerosis; CSF = cerebrospinal fluid; AQP4-Ab = aquaporin-4 antibody; ns = not significant.

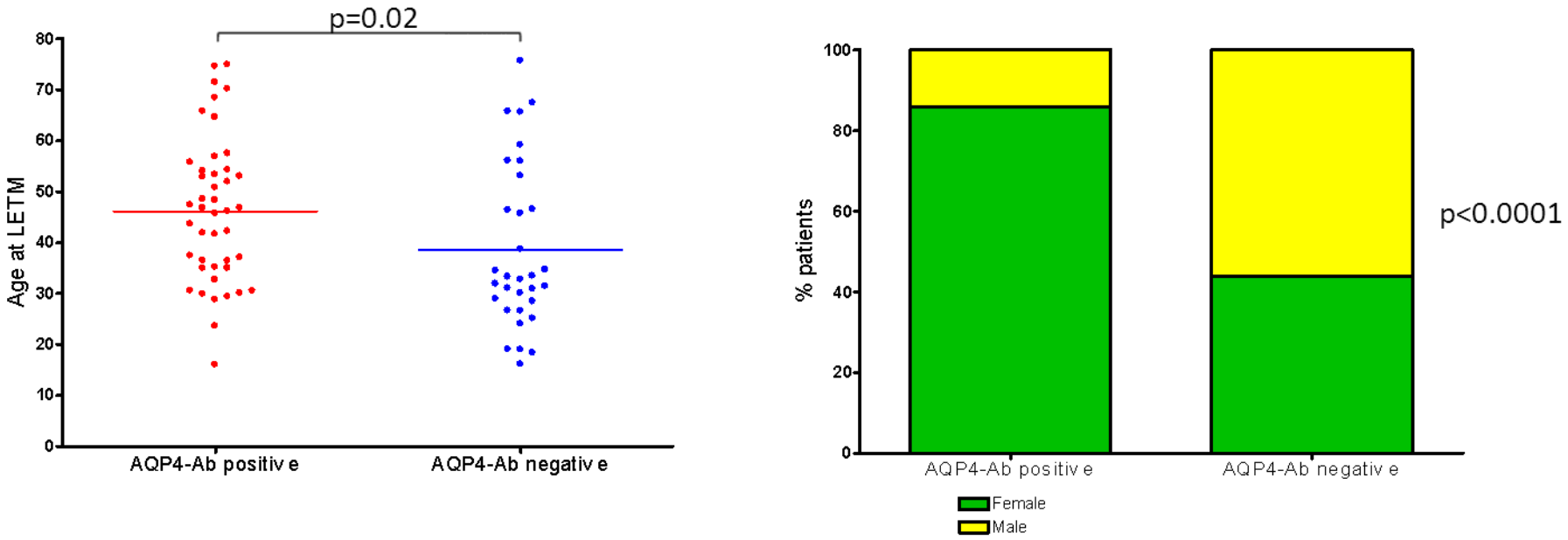


Figure 5.1. Age and gender differences between AQP4-Ab positive and negative patients. AQP4-Ab positive patients were significantly older at the time of LETM than AQP4-Ab negative patients (mean 45.3 ± 14.8 vs. 37.7 ± 16.1; p=0.02) and comprised a significantly higher proportion of females (86% vs. 44%; p<0.0001).

then motor disturbance). However initial urinary retention occurred more frequently in AQP4-Ab negative patients; all subsequently developed limb weakness and sensory disturbance. LETM attacks tended to be associated with severe disability in both groups. The severity as measured by the EDMUS score was similar at nadir and at maximum recovery (which included only patients with follow up duration >6 months) in the AQP4-Ab positive and negative groups, and thus the median improvement (i.e. median change in EDMUS) was similar [2 (0 to 7) vs. 3 (range 0 to 8)]. The proportions of patients left unable to walk 100 metres unaided (EDMUS $\geq$ 6) and wheelchair dependent (EDMUS $\geq$ 8) were also similar.

5.3.3 Treatment in AQP4-Ab positive and negative LETM patients

Acute treatment was comparable in the two groups. Thirty four (77%) AQP4-Ab positive patients and 25 (78%) AQP4-Ab negative patients were treated with methylprednisolone and 11 (25%) AQP4-Ab positive patients and six (19%) AQP4-Ab negative patients additionally underwent plasma exchange. More AQP4-Ab positive patients were started on long-term immunosuppression following the LETM attack than AQP4-Ab negative patients (41% vs. 13%; p=0.005).

5.3.4 Disease course in AQP4-Ab positive and negative LETM patients

Patients with positive AQP4-Ab antibodies were significantly more likely to experience another episode of CNS inflammation after their LETM (74% vs. 31%; p=0.001), but this may be partly related to the longer follow-up period. Allowing for this, by performing survival analysis, showed a trend towards greater probability of relapsing over time in the AQP4-Ab positive group (p=0.069; Figure 5.2) although the likelihood of early relapse (in the first 18 months) appeared similar. Most relapsing patients had a further LETM but six AQP4-Ab positive patients and one AQP4-Ab negative patient went on to have ON. One AQP4-Ab positive patient died from respiratory failure during their first LETM attack and a further four patients (all AQP4-Ab positive) have subsequently died.

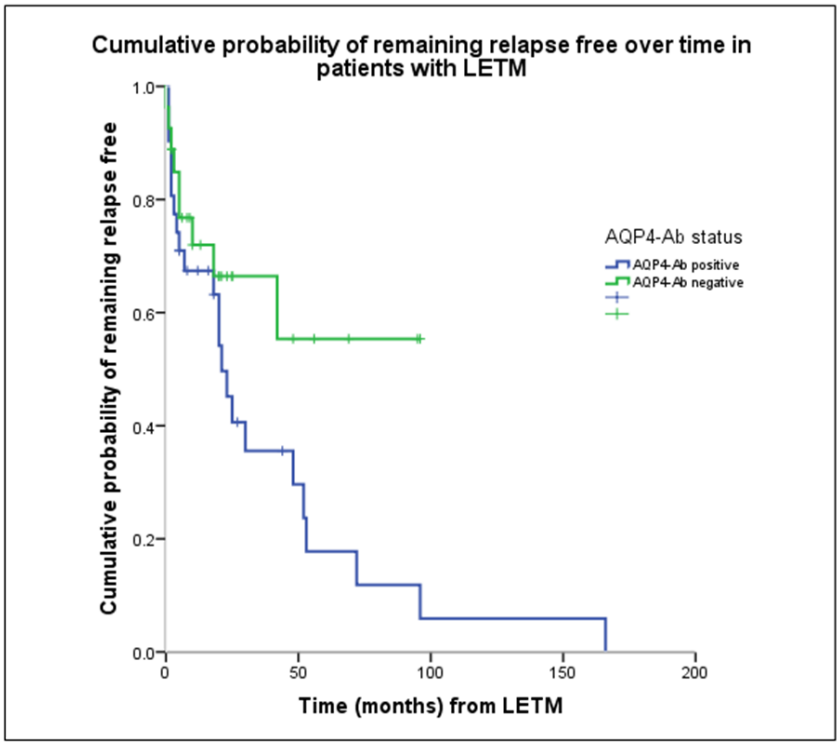


Figure 5.2. Risk of relapse in AQP4-Ab positive and negative LETM patients. There was a trend towards greater probability of relapsing over time in patients with positive AQP4-Ab compared to patients without AQP4-Ab (p=0.069) although the likelihood of early relapse (in the first 18 months) appeared similar.

5.3.5 MRI findings in AQP4-Ab positive and negative LETM patients

Sixty two spinal MRI scans from the acute attack were available for review (Table 5.1). The mean length of the longest lesions did not differ between AQP4-Ab positive and AQP4-Ab negative patients and there was no correlation between lesion length and nadir or maximum recovery EDMUS scores. However, of possible practical significance, involvement of the conus was significantly more common in AQP4-Ab negative patients ($p<0.01$). MRI brain scans from the acute attack were available from 61 patients and there were no differences in the proportions of patients with normal scans, non-specific white matter lesions or MS-like lesions between the two groups (Table 5.1). Two AQP4-Ab negative patients with MS-like lesions fulfilled McDonald criteria for dissemination in space but there was no dissemination in time and the distribution of lesions was more typical for ADEM, with involvement of both white and grey matter.

5.3.6 CSF findings in AQP4-Ab positive and negative LETM patients

CSF results from the acute attack were available from 44 patients (Table 5.1). CSF OCBs at presentation were rare and only present in the AQP4-Ab positive patients and there was a trend towards more pleocytosis in the negative group.

5.3.7 Diagnoses in patients testing negative for AQP4-Ab

Since AQP4-Ab are highly specific for NMO/NMOSD, patients testing positive for this antibody were assumed to have AQP4-Ab mediated NMO/NMOSD. Most of the AQP4-Ab negative patients had an alternative identifiable cause (Figure 5.3).

5.3.7.1 LETM with other identifiable causes

Six patients tested positive for MOG-Ab. These patients will be described in detail in chapter 7. Other diagnoses in AQP4-Ab negative LETM patients included CNS vasculitis (n=1), a leptomeningeal syndrome associated with connective tissue disease (n=1), infection (n=4; one tuberculosis, one mycoplasma, one Epstein Barr virus, one West Nile virus), paraneoplastic disease due to mesothelioma (n=1) and spinal cord infarction (n=1). Additionally, five patients had

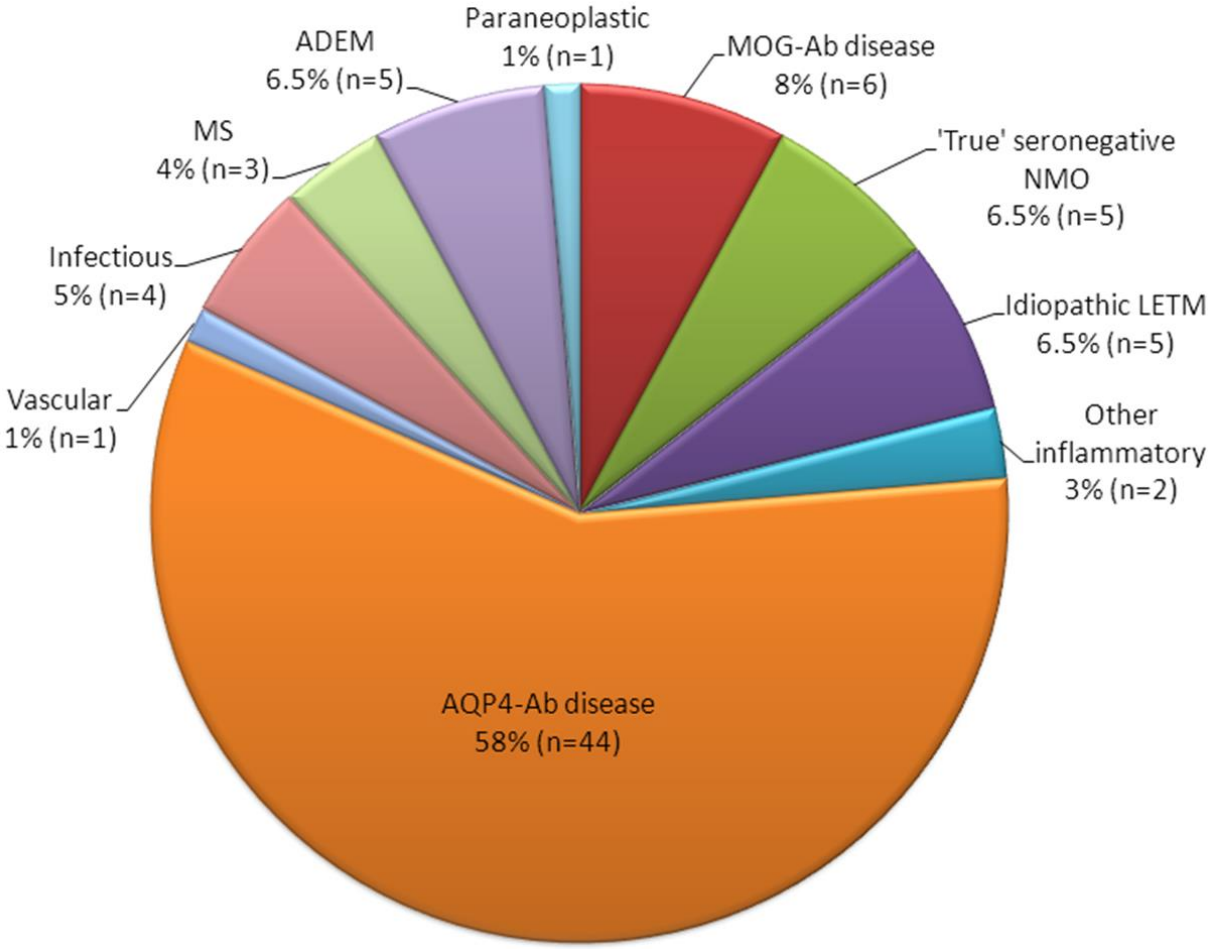


Figure 5.3. Diagnoses in LETM patients. Fifty eight per cent of patients with LETM were positive for AQP4-Ab. Alternative diagnoses could be identified in the majority of the remaining patients; these included MOG-Ab disease, infection and other inflammatory disorders. As all patients were referred to our specialist NMO service, the proportion of patients with inflammatory causes is likely to be over-represented.

monophasic multifocal inflammation of the CNS consistent with ADEM, and three patients subsequently experienced a relapsing-remitting illness that was compatible with MS. Infectious aetiologies were identified through high clinical suspicion e.g. relevant foreign exposure, together with positive serology. Diagnoses of ADEM and MS were made on clinical grounds with supporting paraclinical criteria. Patients diagnosed with ADEM had a preceding illness and/or supportive symptoms such as lethargy, irritability, headache, fever and nausea, and have remained relapse-free off treatment during the follow-up period. Patients diagnosed with MS had relatively short LETM (3 or 4 vertebral bodies in length), had MRI brain findings suggestive of, though not diagnostic of, MS at presentation and/or at follow-up and experienced further relapses typical of MS over the follow-up period that included myelitis with short cord lesions or brain/brainstem attacks. One had a strong family history of MS and has entered a secondary progressive course. CSF was performed later in the disease course in one patient and was positive for OCBs.

5.3.7.2 Seronegative NMO and idiopathic LETM

In 10 patients, despite extensive investigations that included a full autoimmune, infection and metabolic screen, no cause for the LETM could be identified. Five of these patients had also experienced at least one ON attack and fulfilled the Wingerchuk revised diagnostic criteria for NMO and were felt to have seronegative NMO and five had disease limited to the spinal cord and were termed idiopathic LETM.

The five seronegative NMO patients all had relapsing disease. They had a similar female preponderance (4:1) and similar mean age at first LETM (38.2 ± 17.1 years) compared to AQP4-Ab positive NMO patients, although they comprised a higher proportion of Caucasian patients (100% vs. 60%; p=0.014). The proportions of patients with co-existing autoimmunity were also similar (20% seronegative vs. 27% seropositive). There were no obvious differences in imaging or CSF characteristics compared to AQP4-Ab positive NMO patients and no differences between nadir (median 7.25) and maximum recovery EDMUS (median 3) scores.

The five patients with idiopathic LETM comprised patients with monophasic (n=2, follow-up duration 6 and 95 months; no immunosuppression received) and relapsing LETM (n=3, follow-up duration 15, 31 and 71 months). These patients included a significantly higher proportion of males compared to AQP4-Ab positive patients (80% vs. 14%; p=0.004) but had a similar mean age at onset (45.6 ± 20.2 years); the mean lesion length on MRI was comparable to that seen in group overall (7.2 ± 3.03 vertebral segments).

5.3.7.3 Patients fulfilling the 2006 Wingerchuk diagnostic criteria for NMO

Thirty five of 76 patients (46%) in the cohort fulfilled the 2006 Wingerchuk NMO diagnostic criteria and would be included in most clinical studies of NMO. However, only 20 of these were AQP4-Ab positive; 15 were AQP4-Ab negative. A comparison of these two groups is shown in Table 5.2, with a shorter follow-up time, younger age, and greater likelihood of a 'classic Devic's' presentation (i.e. simultaneous ON and TM) in the AQP4-Ab negative group. However, the AQP4-Ab negative group comprised a mixed group of diagnoses, including four MOG-Ab positive patients, three with ADEM, and three with MS, with only one third actually felt to have relapsing seronegative NMO (Figure 5.4).

Table 5.2. Comparisons between AQP4-Ab positive and negative patients fulfilling current NMO diagnostic criteria

	AQP4-Ab positive (n=20)	**AQP4-Ab negative (n=15)**	**P value**
Number (%) females	16 (80%)	9 (60%)	ns
Mean age at LETM ± s.d.	40.7 ± 13.0	30.9 ± 12.0	0.040
Caucasian ethnicity [n(%)]	12 (60%)	12 (80%)	ns
Median FU since LETM, months (range)	84.1 (12.6 to 260.2)	33.0 (9.3 to 169.4)	0.016
Median FU since disease onset, months (range)	113.6 (17.4 to 260.8)	56.3 (9.3 to 214.1)	0.017
Devic's phenotype at onset [n(%)]	2 (10%)	8 (53%)	0.008
Median nadir EDMUS score (range)	7.0 (3 to 9)	8.0 (3 to 8)	ns
Median EDMUS score at recovery (range)	2.0 (0 to 6)	2.0 (0 to 7)	ns
% relapsing within one year of LETM (including only those with ≥12 months FU)	14 of 20 (70%)	6 of 14 (43%)	ns

LETM = longitudinally extensive transverse myelitis; s.d. = standard deviation; n = number; FU = follow-up; EDMUS = European Database System for Multiple Sclerosis; AQP4-Ab = aquaporin-4 antibody; ns = not significant.

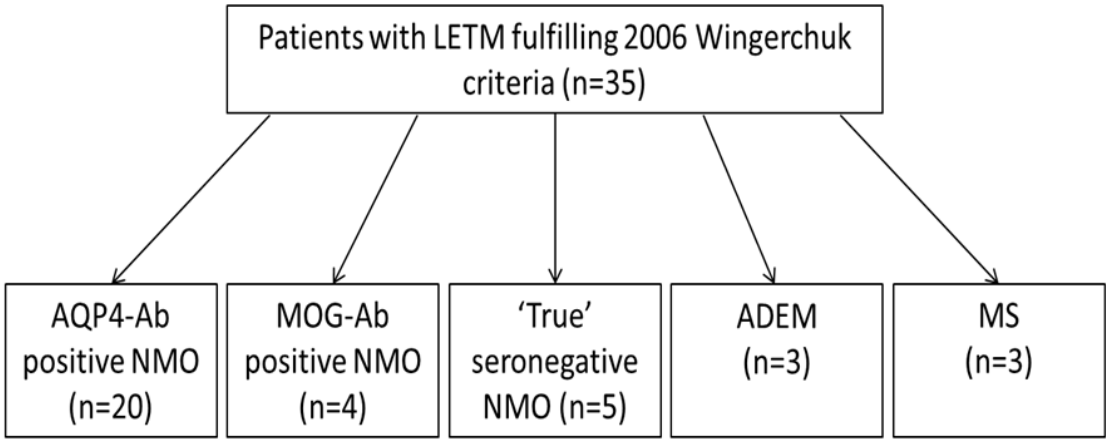


Figure 5.4. Diagnoses in NMO patients fulfilling the 2006 NMO clinical diagnostic criteria. The 2006 Wingerchuk NMO diagnostic criteria were not specific for NMO and patients fulfilling these criteria often had alternative diagnoses.

5.4 Discussion

It has become clear since the discovery of AQP4-Ab that the spectrum of NMO encompasses patients with disease isolated to the optic nerves, or more commonly the spinal cord. Studies looking at differences in demographic and clinical features between AQP4-Ab positive and negative NMO patients have largely compared patients with only the full NMO phenotype (Bizocco, Lolli et al, 2009; Cabrera-Gomez, Kurtzke et al, 2009b; Adoni, Lino et al, 2010; Akman-Demir, Tüzün et al, 2011; Nagaishi, Takagi et al, 2011; Zhong, Wang et al, 2012). These studies have yielded conflicting data (Table 5.3), which may in part be due to the fact that most used a tissue IIF assay to define cohorts (Bizocco, Lolli et al, 2009; Cabrera-Gomez, Kurtzke et al, 2009b; Adoni, Lino et al, 2010; Zhong, Wang et al, 2012). As mentioned, this assay misses up to 25% of AQP4-Ab positive cases compared to CBAs (Waters, Jarius et al, 2008; Chan, Kwan et al, 2010; Waters, McKeon et al, 2012) and therefore it is likely that these studies included false negative patients which may have diluted any differences. Using sensitive cell-based and FACS AQP4-Ab assays to define AQP4-Ab positive and negative patients we have described alternative diagnoses in patients with AQP4-Ab negative LETM/NMO and identified features of LETM that can help differentiate patients with AQP4-Ab from those without AQP4-Ab in the acute setting, when the final diagnosis is often not apparent (Kitley, Leite et al, 2013). We did not include AQP4-Ab negative ON patients because this commonly occurs in the context of MS, which would have dominated the phenotype and additionally these patients commonly present to eye departments and the small numbers of patients seen within our service are not representative of this group as a whole.

Age at LETM and gender were discriminators between AQP4-Ab positive and AQP4-Ab negative patients. AQP4-Ab positive patients were significantly older and showed a strong female preponderance. Older females aged >45 years at LETM had an 88% chance of being AQP4-Ab positive whereas young males aged <35 years at LETM had an 82% chance of being AQP4-Ab negative. Older onset age and stronger female preponderance were noted to be discriminators between relapsing and monophasic NMO patients even before the discovery of AQP4-Ab

Table 5.3. Studies comparing features of AQP4-Ab positive and negative NMO/NMOSD patients

Study	N	AQP4-Ab +ve	Demographic differences	Clinical differences	Paraclinical differences
Bizzoco, Lolli et al, 2009	13	69%	No gender difference; similar onset age	More relapses and higher final EDSS scores in seropositive group	No differences in presence of autoantibodies Lower frequency of OCBs in seronegative group
Cabrera-Gomez, Kurtzke et al, 2009b	48	33%	N/A	More relapses and higher EDSS scores in seropositive group	No differences in CSF characteristics Greater number of brain lesions and spinal grey matter or holocord lesions in seropositive group
Adoni, Lino et al, 2010	28	64%	No gender difference; similar onset age	No difference in EDSS scores between the groups	No differences in presence of autoantibodies No difference in presence or length of LETM
Akman-Demir, Tüzün et al, 2011	35	60%	No gender difference; similar onset age	No differences in number of relapses or proportion with relapsing course Higher final EDSS in seropositive group and greater proportion significantly disabled (EDSS >6)	No difference in proportion with OCBs
Nagaishi, Takagi et al, 2011	401	95%	Higher proportion of males and younger onset age in seronegative group	No difference in onset relapse type, ARR or EDSS at last follow-up Significantly more seronegative patients had severe visual impairment at last follow-up	Higher proportion with positive anti-nuclear antibodies in seropositive group No MRI differences No difference in proportion with OCBs
Jarius, Ruprecht et al, 2012	175	78%	Higher proportion of males in seronegative group; similar onset age	Simultaneous ON+TM and bilateral ON at onset and monophasic course more common in seronegative group No differences in ARR or annualised EDSS progression index Significantly more seropositive patients had severe attacks	Autoimmune diseases and autoantibodies more common in seropositive group No differences in CSF characteristics Trend towards longer cord lesions in seropositive patients
Zhong, Wang et al, 2012	52	85%	Higher proportion of males in seronegative group; similar onset age	No differences in ARR, EDSS at last follow-up or proportion of patients reaching EDSS scores at last follow-up	Central cord lesions more common and trend towards longer cord lesions in seropositive group

N = number; AQP4-Ab = aquaporin-4 antibody; +ve = positive; N/A = not available; EDSS = Expanded Disability Status Scale; ARR = annualised relapse rate; ON = optic neuritis; TM = transverse myelitis; VA = visual acuity; OCBs = oligoclonal bands; CSF = cerebrospinal fluid; MRI = magnetic resonance imaging; LETM = longitudinally extensive transverse myelitis.

(Wingerchuk, Hogancamp et al, 1999); it is likely that many of the monophasic patients in this report were AQP4-Ab negative. Other features that differed between AQP4-Ab positive and negative patients included a higher frequency of co-existing autoimmune disorders in AQP4-Ab positive patients, as has been described previously (Nagaishi, Takagi et al, 2011; Jarius, Ruprecht et al, 2012).

As mentioned previously, several characteristic clinical features of AQP4-Ab disease have recently been described. These include intractable vomiting and/or hiccups (Apiwattanakul, Popescu et al, 2010; Riphagen, Modderman et al, 2010) and pain and tonic spasms (Kim, Go et al, 2012; Qian, Lancia et al, 2012). We found that although there was a trend towards preceding vomiting being more common in AQP4-Ab positive patients, this did not reach significance; AQP4-Ab status did not predict pain in LETM.

Attack severity and outcomes were not related to AQP4-Ab status, and although similar acute treatments were used in the two groups, we cannot rule out the possibility that lack of differences in disability outcomes may have been due to initiation of long-term immunosuppression in the AQP4-Ab positive group. The follow-up time in AQP4-Ab negative patients was shorter than in AQP4-Ab positive patients, making it difficult to draw definitive conclusions about risk of relapse over time in the two groups, but there was a trend towards higher risk of relapsing disease in AQP4-Ab positive patients, which has been shown previously (Rivera, Kurtzke et al, 2008).

One of the hallmarks of NMO with AQP4-Ab is the longitudinally extensive nature of spinal cord lesions. However, in agreement with previous studies (Adoni, Lino et al, 2010; Jarius, Ruprecht et al, 2012; Zhong, Wang et al, 2012), we found that the length of cord lesions in AQP4-Ab negative LETM patients is no different from those with AQP4-Ab. However, we have shown that conus involvement occurs more commonly in AQP4-Ab negative LETM and this may account for the fact that urinary retention as the presenting neurological feature was only seen in these patients. A recent study (Chang, Lyu et al, 2012) found that central grey matter lesions were more frequent in patients with AQP4-Ab than in seronegative patients. Thus, there may be a number of radiological

clues to differentiate AQP4-Ab positive and negative LETM, which warrant validation in larger studies.

As reported in chapter 3, it is rare to see a 'classic' monophasic Devic's syndrome [as originally described (Devic, 1894)] with simultaneous ON and LETM as the onset attack in AQP4-Ab positive NMO/NMOSD. Higher rates have been reported in clinical NMO studies that included seronegative patients (Rivera, Kurtzke et al, 2008; Bichuetti, Oliveira et al, 2009; Collongues, Marignier et al, 2010; Akman-Demir, Tüzün et al, 2011) and differences between monophasic NMO patients and those with recurrent attacks were eluded to even before the discovery of AQP4-Ab (Wingerchuk, Hogancamp et al, 1999; Wingerchuk, Weinshenker et al, 2003). Here we have found a monophasic Devic's syndrome to be significantly more common in AQP4-Ab negative NMO patients, which is in concordance with other studies (Akman-Demir, Tüzün et al, 2011; Jarius, Ruprecht et al, 2012). Thus, we feel that AQP4-Ab negative patients with a monophasic Devic's syndrome are different from relapsing NMO patients with AQP4-Ab and should not be included in future clinical NMO studies. We have found MOG-Ab in some of these patients and although the pathogenicity of MOG-Ab has not yet been proven, in our experience MOG-Ab positive patients have a characteristic phenotype (see chapter 7) and we believe these patients should be classed separately. However, further studies are required to characterise the role of MOG-Ab.

In addition to demonstrating useful differences between AQP4-Ab positive and negative patients, we also found that many AQP4-Ab negative LETM and NMO patients had alternative diagnoses. Only 57% of patients fulfilling the current clinical NMO diagnostic criteria were AQP4-Ab positive but many AQP4-Ab negative patients had alternative diagnoses, including infectious and inflammatory aetiologies. Thus, it is vital that patients presenting with LETM are screened for other causes before being labelled as NMO. Vascular causes such as spinal cord infarction and dural fistulae should also be borne in mind. Since this work was performed, we have encountered two patients with the latter diagnosis who were initially treated as inflammatory LETM.

The number of 'true' seronegative NMO patients (relapsing NMO where other causes have been excluded) in this cohort was small and we believe that when good assays are used to define AQP4-Ab status, seronegative NMO is rare. The 'true' AQP4-Ab seronegative patients had similar features to AQP4-Ab positive patients and they may have very low titre AQP4-Ab, below the detection limit of our assay or they may have an alternative antigen target with similar pathogenic consequences. Some patients had received corticosteroids prior to AQP4-Ab testing and we cannot exclude the possibility that this may have produced a false negative result, although in our experience it is rare for AQP4-Ab to become negative following treatment. Despite extensive investigations, the cause of LETM could not be determined in a minority of patients. Idiopathic LETM may be a distinct entity; a recent study found that the HLA-DRB1*13 genotype was over-represented in such patients (Sepulveda, Blanco et al, 2012).

Since the work in this thesis was performed, another large study has been published describing the differences between AQP4-Ab positive and negative NMO patients in cohorts defined using a sensitive CBA (Marignier, Bernard-Valnet et al, 2013). In concordance with our findings, patients without AQP4-Ab were more likely to be male and present with simultaneous or sequential ON and TM. These differences were not apparent when serological status was defined by the less sensitive tissue IIF assay, emphasising that previous studies using such assays are likely confounded by inclusion of false negatives, which dilute any differences.

Our study has several limitations. Firstly, some of the data were collected retrospectively, which means that some data were incomplete. Secondly, all patients had been referred to our specialist Neuro-inflammatory team and thus there may be an element of referral bias and over-representation of inflammatory causes. Thirdly, differentiating ADEM and optico-spinal MS from seronegative NMO cannot be made with absolute certainty in the absence of pathological confirmation. Fourthly, we did not include NMOSD patients with bilateral or recurrent ON as they are commonly looked after by the Neuro-ophthalmology service. Comparison of patients with recurrent ON with or without AQP4-Ab is warranted. Finally, because of the small N values for

some comparisons, p values were not adjusted for multiple comparisons, and larger studies are required to confirm the trends found.

In conclusion, AQP4-Ab negative patients with LETM, even if fulfilling the Wingerchuk NMO diagnostic criteria, have a high chance of having alternative diagnoses. Using sensitive AQP4-Ab assays, seronegative NMO is rare and patients testing negative for AQP4-Ab using such assays should not be labelled as NMO/NMOSD without first pursuing an alternative cause. In this clinically and immunologically well-defined cohort, we have found features that may distinguish between AQP4-Ab positive and negative LETM patients in the acute setting. AQP4-Ab negative patients are younger than those with AQP4-Ab, are more likely to present with a classic Devic's syndrome and are more likely to have conus involvement and early urinary retention. Clinically, AQP4-Ab seronegative NMO/NMOSD patients represent a diverse group and in the following chapter, the immunological characteristics of sera from these patients will be studied to investigate whether they are also heterogeneous.

Chapter 6: Alternative antigen targets in seronegative NMO/NMOSD

6.1 Introduction

Since the discovery of AQP4-Ab, it has been recognised that a proportion of patients with clinical features of NMO/NMOSD are seronegative for AQP4-Ab. Seronegative NMO has generated much interest because lack of a biomarker makes diagnosis and management of these patients difficult.

Many clinicians perceive these patients to be identical to those with AQP4-Ab, and manage them in the same way with aggressive long-term immunosuppression. However, as discussed in chapter 5, there do appear to be differences between patients with and without AQP4-Ab and it is possible that seronegative NMO patients have different aetio-pathogenic mechanisms.

The reasons for AQP4-Ab seronegativity are likely multiple, as has been shown in other autoimmune disorders such as MG. Firstly, it is possible that some patients have very low levels of AQP4-Ab, below the detection limit of current assays. This is supported by the fact that the original NMO-IgG tissue IIF assay misses up to 25% of AQP4-Ab positive cases compared to newer CBAs, and it is likely that even current assays have not yet reached maximum sensitivity. Secondly, some seronegative NMO patients may have antibodies against an alternative antigenic target(s). Thirdly, it is possible that some patients do not have an autoantibody mediated disease but that disease in these patients is driven by other mechanisms such as T cell mediated inflammation. Finally, as was shown in chapter 5, it is likely that some patients have alternative diagnoses.

6.1.1 Aims

The main aims of this chapter were:

- To determine whether some seronegative NMO/NMOSD patients have low titre AQP4-Ab
- To investigate whether seronegative NMO/NMOSD patients have an astrocytopathy
- To investigate potential alternative antigenic targets in seronegative NMO/NMOSD

6.2 Methods and technical approaches

Ninety six serum samples from 62 patients with clinical features suggestive of NMO/NMOSD, but negative for AQP4-Ab on the Oxford laboratory routine diagnostic CBA were used for this part of the work. The patients were all known to the clinical NMO service at the John Radcliffe Hospital, Oxford or The Walton Centre, Liverpool and samples were obtained prior to January 2011. Control sera were used from 37 AQP4-Ab positive (on routine diagnostic CBA) NMO/NMOSD patients known to the Oxford NMO service, 27 local clinically well-characterised MS patients, 18 MG patients and 11 healthy controls.

6.3 Results

6.3.1 Clinical phenotype of the seronegative NMO/NMOSD patients

The clinical phenotype of the patients whose sera were used for experiments are summarised in Table 6.1.

6.3.2 Presence of low titre AQP4-Ab in seronegative NMO/NMOSD

All seronegative NMO/NMOSD patients in the cohort were negative for AQP4-Ab on the Oxford routine diagnostic CBA at a standard serum dilution of 1:20. However, it was recently shown that an in-house FACS assay developed by Dr Patrick Waters had slightly superior sensitivity to the Oxford CBA and assays used in other large Neuro-immunology centres (Waters, McKeon et al, 2012). Thus, the seronegative NMO/NMOSD cohort samples were first screened for AQP4-Ab on FACS to investigate whether some may have low titre AQP4-Ab.

A positive score was considered to be one greater than three s.d. above the mean of the control cohort (equivalent to a score of 38). Four samples from three seronegative NMO/NMOSD patients were low positive [Figure 6.1; NMO 128 (1 of 2 samples), L-01 (1 of 2 samples) and L-04 (2 of 2 samples)]. However, one of 27 MS control samples and one of 18 MG control samples were also low positive (Figure 6.1).

Table 6.1. Clinical phenotype of seronegative NMO/NMOSD patients whose sera were used for experimental work

Phenotype	No. of patients
Relapsing NMO (attacks of both ON + TM)	16
Monophasic NMO (simultaneous or rapidly sequential ON + TM)	8
Relapsing LETM	10
Monophasic LETM	13
Relapsing ON	5
Monophasic severe or bilateral ON	3
Optico-spinal MS	7

NMO = neuromyelitis optica; ON = optic neuritis; TM = transverse myelitis; LETM = longitudinally extensive transverse myelitis; MS = multiple sclerosis; No. = number.

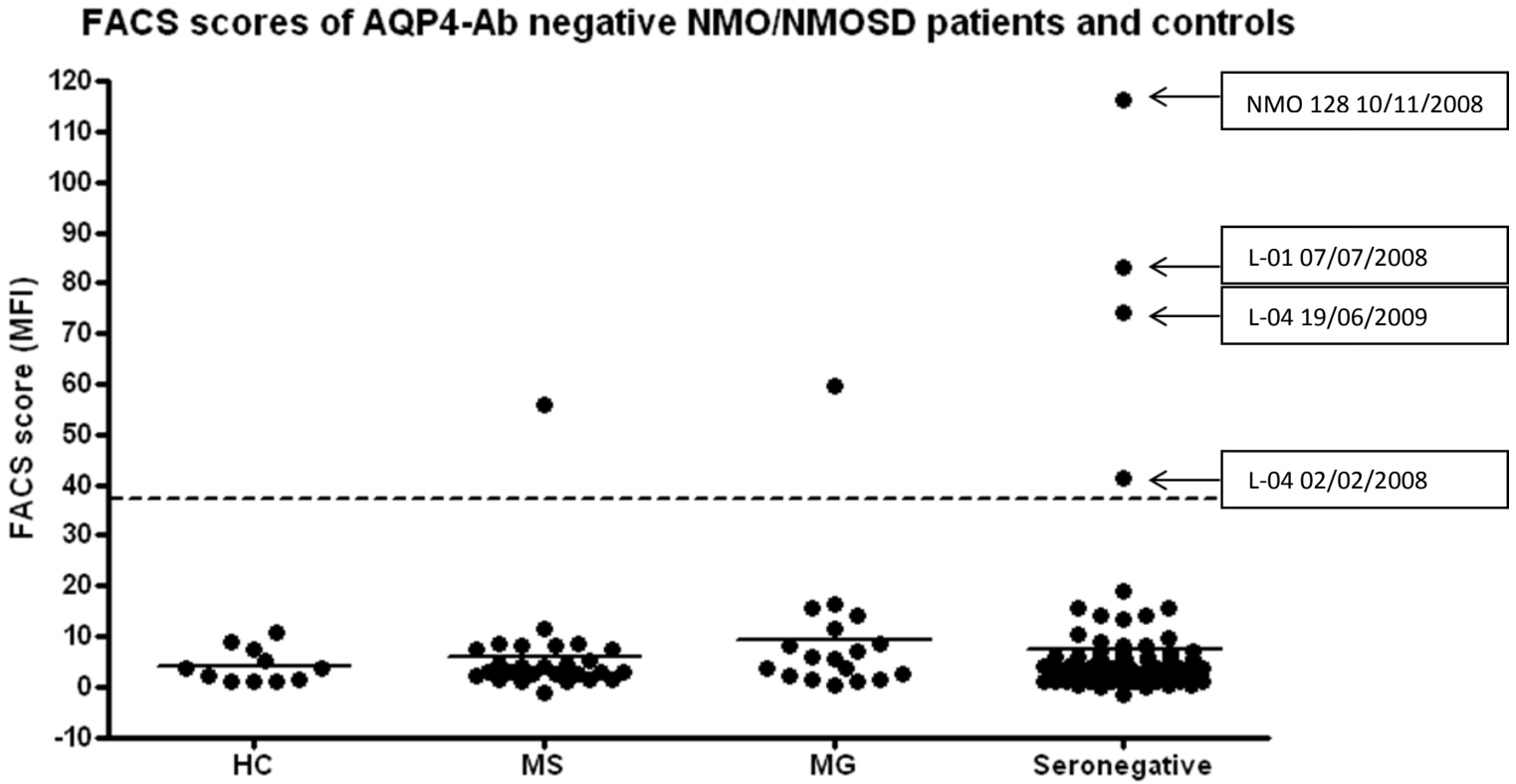


Figure 6.1. FACS scores of seronegative NMO/NMOSD patients and controls. FACS identified four samples from three seronegative NMO/NMOSD patients who were low positive for AQP4-Ab. However, one MS patient and one MG patient were also low positive. HC = healthy controls; MS = multiple sclerosis disease controls; MG = myasthenia gravis disease controls; seronegative = seronegative NMO/NMOSD.

NMO128 has relapsing ON and brainstem disease with a brain MRI scan typical for NMO, whilst L-01 had a monophasic LETM with the low positive sample being taken during the acute attack before treatment; a later remission sample taken on immunosuppression tested negative. L-04 has relapsing ON and LETM; both samples were taken during remission whilst on treatment. The MS control testing low positive has mild relapsing-remitting MS with no characteristics suggestive of NMO, and the MG control testing low positive also has no features of NMO.

To investigate whether these samples represented true low positives, FACS experiments were repeated using a higher concentration of patient sera (1:5 dilution instead of standard 1:10), and using three times the normal volume of serum (60 µl in 240 µl of FACS buffer instead of standard 20 µl in 80 µl). Neither of these techniques increased the mean FACS score of any of the samples. However, due to high clinical suspicion of a diagnosis of AQP4-Ab disease over follow-up, the sample from NMO 128 was retested on the routine diagnostic CBA and was low positive at the standard 1:20 dilution, and clearly positive at 1:5 dilution. Additionally, serum from this patient was able to immunoprecipitate AQP4 from AQP4-transfected cells (see later). This patient was therefore felt to genuinely be AQP4-Ab positive.

6.3.3 Screening for known CNS antigens in seronegative NMO/NMOSD

6.3.3.1 Myelin oligodendrocyte glycoprotein antibodies

MOG-Ab have recently been identified in some patients with inflammatory demyelinating disorders of the CNS, particularly in paediatric ADEM. Therefore, we investigated whether some of the seronegative NMO/NMOSD patients, especially those with a monophasic 'ADEM-like' phenotype, may have antibodies to MOG. All seronegative NMO/NMOSD and all control samples were screened for MOG-Ab using a CBA, in which HEK293 cells were transiently transfected with the extracellular domain of human MOG, together with eGFP. Samples scoring 0.5 or above were repeated and samples scoring ≥ 1 on two occasions were considered positive. Some of the samples were tested by Dr Mark Woodhall during development of the assay and some samples were screened by myself after the assay had been validated. Six of the 63 (9.5%) AQP4-Ab

seronegative NMO/NMOSD patients were positive for MOG-Ab (Figure 6.2). Three of these patients had a monophasic NMO phenotype and two had monophasic LETM; these patients were seen within the Oxford clinical NMO service, and the clinical features of these patients, together with four more MOG-Ab positive Oxford patients identified more recently, are described in chapter 7. One patient, followed in Liverpool is reported to have a relapsing NMO phenotype though relapses were mild and not radiologically proven. None of 37 AQP4-Ab positive NMO/NMOSD patients, 11 healthy controls, 18 MG controls or 27 MS controls were positive for MOG-Ab.

MOG-Ab were shown by Dr Woodhall to be predominantly of the IgG1 subtype and capable of activating complement on the cell surface membranes of HEK293 cells transfected with MOG. Additionally, where measured longitudinally, MOG-Ab titres decreased over time following clinical recovery, compatible with a pathogenic role (Kitley, Woodhall et al, 2012).

None of the MOG-Ab positive samples were found to bind to oligodendrocytes in primary cultures, though cultures were not specifically set up to grow oligodendrocytes (i.e. oligodendrocyte differentiation media which enables myelination was not used).

6.3.3.2 Glycine receptor antibodies

GlyR-Ab have been found incidentally in some patients whose sera were sent for diagnostic AQP4-Ab testing (A. Vincent, personal communication); presumably therefore these patients have some features of NMO. The significance of these antibodies in such patients has not been investigated. Given this finding, the anatomical distribution of GlyR, and the fact that GlyR-Ab are pathogenic in other situations, seronegative NMO/NMOSD patients were screened for GlyR-Ab.

All seronegative NMO/NMOSD and all control samples were screened for GlyR-Ab (co-transfection with all five subtypes plus eGFP) using a CBA. Samples scoring 0.5 or above were repeated and samples scoring ≥ 1 on two occasions were considered positive. Four samples were low positive (mean CBA score of 1 or 1.5 from two separate experiments; Figure 6.3).

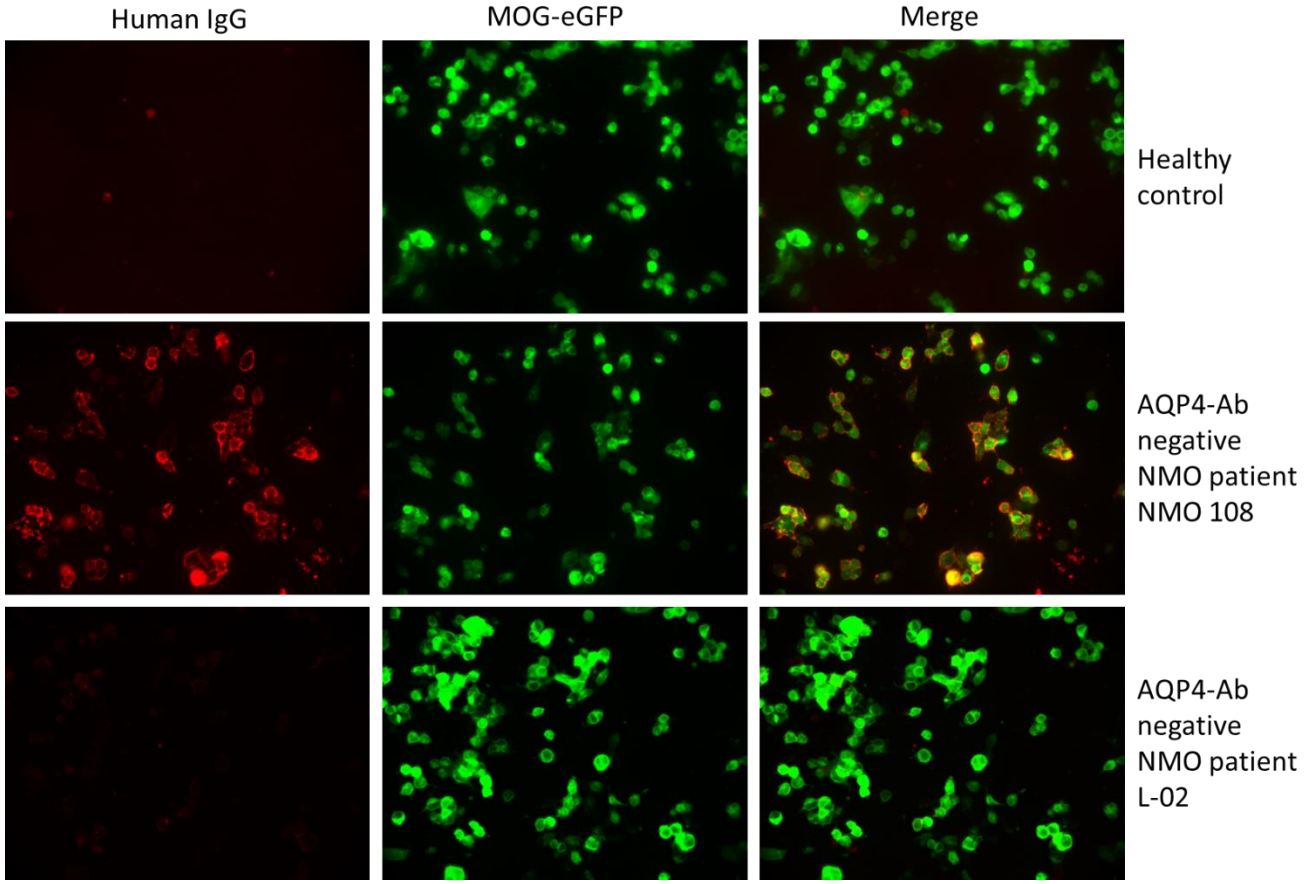


Figure 6.2. Binding of seronegative NMO/NMOSD patient sera to MOG-transfected cells. MOG-eGFP transfected HEK293 cells are shown in green, and detection of patients' IgG identified with red-anti-human IgG. Serum from a healthy control does not show any IgG binding to MOG-eGFP expressing cells (top panel). Middle and bottom panels show examples of a strongly MOG-Ab positive seronegative NMO sample (NMO 108; middle panel) and a low MOG-Ab positive seronegative NMO sample (L-02, bottom panel). X40 magnification.

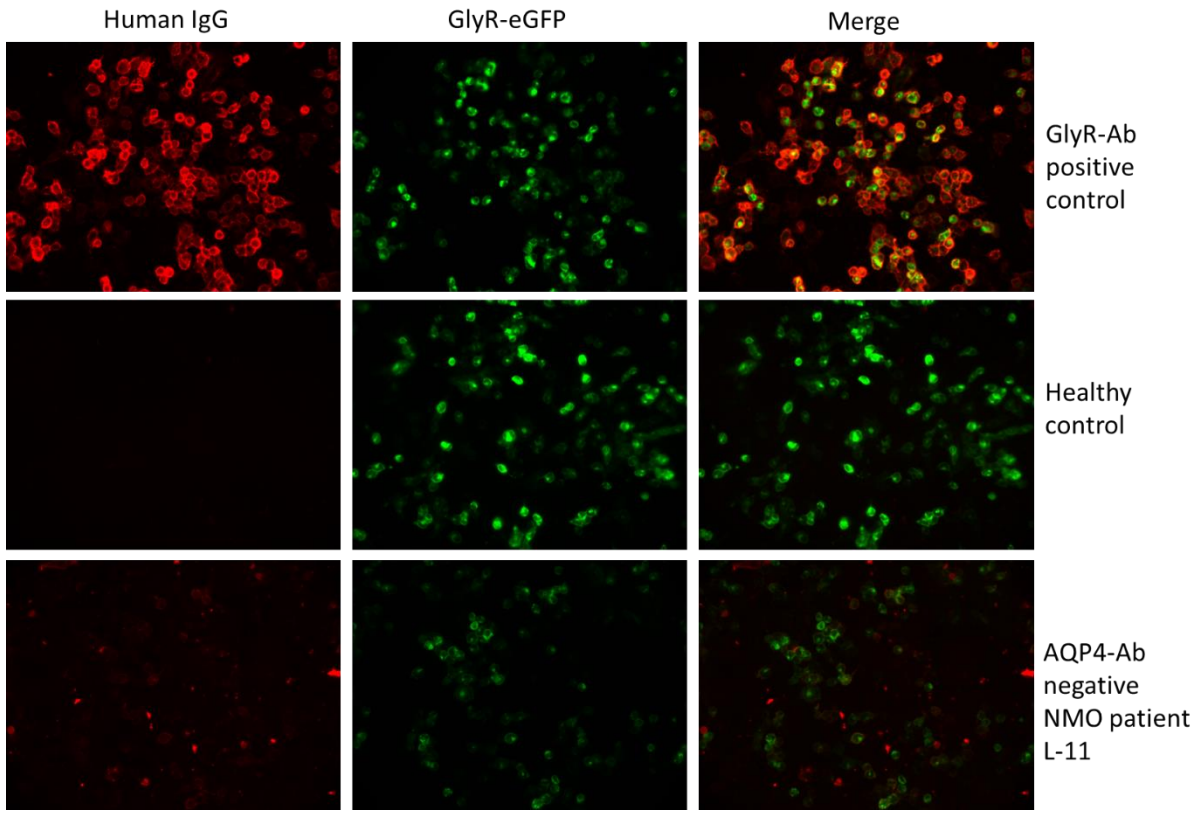


Figure 6.3. Binding of NMO/NMOSD patient sera to GlyR-transfected cells. Cells were co-transfected with eGFP and all five glycine receptor subunits and are shown in green, and detection of patients' IgG identified with red-anti-human IgG. Right hand column shows merge. Top panel shows a strong positive control from a patient with the syndrome of progressive encephalomyelitis, rigidity and myoclonus. Serum from a healthy control does not show any IgG binding to GlyR-eGFP expressing cells (middle panel). Bottom panel shows a low GlyR-Ab positive seronegative NMO sample (patient L-11). X40 magnification.

Two of these were strongly AQP4-Ab positive (CBA score 4), one was strongly MOG-Ab positive (CBA score 4) and one was an AQP4-Ab seronegative NMO patient (L-11, monophasic NMO, Liverpool patient).

6.3.4 Alternative astrocytic targets in seronegative NMO/NMOSD

It was next investigated whether sera from seronegative patients bound to astrocytes in primary glial cell cultures. AQP4-Ab positive patient sera is known to bind to astrocytes *in vitro* and if seronegative patients have the same disease with astrocyte pathology, then sera from seronegative patients should bind to astrocytes, even if the target antigen is not AQP4. Primary mixed glial cell cultures established from neonatal (P0-4) rat brains were grown in 24-well plates for five or six days before being incubated with individual patient sera and immunostained for binding of human IgG. Sera from AQP4-Ab positive patients were used as positive controls and to optimise the assay, and sera from MG and MS patients and healthy controls were used as negative controls.

6.3.4.1 Human IgG from AQP4-Ab positive patients binds to astrocytes in vitro

When glial cells were incubated for one hour at room temperature with sera from AQP4-Ab positive NMO/NMOSD patients (1:100 dilution), 21 of 37 (57%) patient samples demonstrated reproducible binding to astrocytes. Binding of human-IgG, detected using a green fluorescent anti-human IgG antibody, was seen as a 'halo' outlining astrocytes, which were visualised with an anti-GFAP antibody (Figure 6.4A). GFAP is an intracellular cytoskeletal protein whereas AQP4 is found on astrocyte cell surface membranes; this is presumably why binding of human IgG extended beyond the boundaries of GFAP staining. Binding of IgG in AQP4-Ab positive patient sera to astrocytes was found to co-localise with AQP4 using a rabbit polyclonal anti-AQP4 commercial antibody (Figure 6.4B). However, in subsequent experiments, anti-GFAP rather than anti-AQP4 was used to visualise astrocytes because a much lower concentration was required.

Immunostaining using astrocytes cultured for more than six days led to reduced specificity of the assay. Increasing the concentration of patient sera to 1:20 or 1:50 did not increase sensitivity and led to an unacceptable amount of background staining. Increasing primary incubation time to two

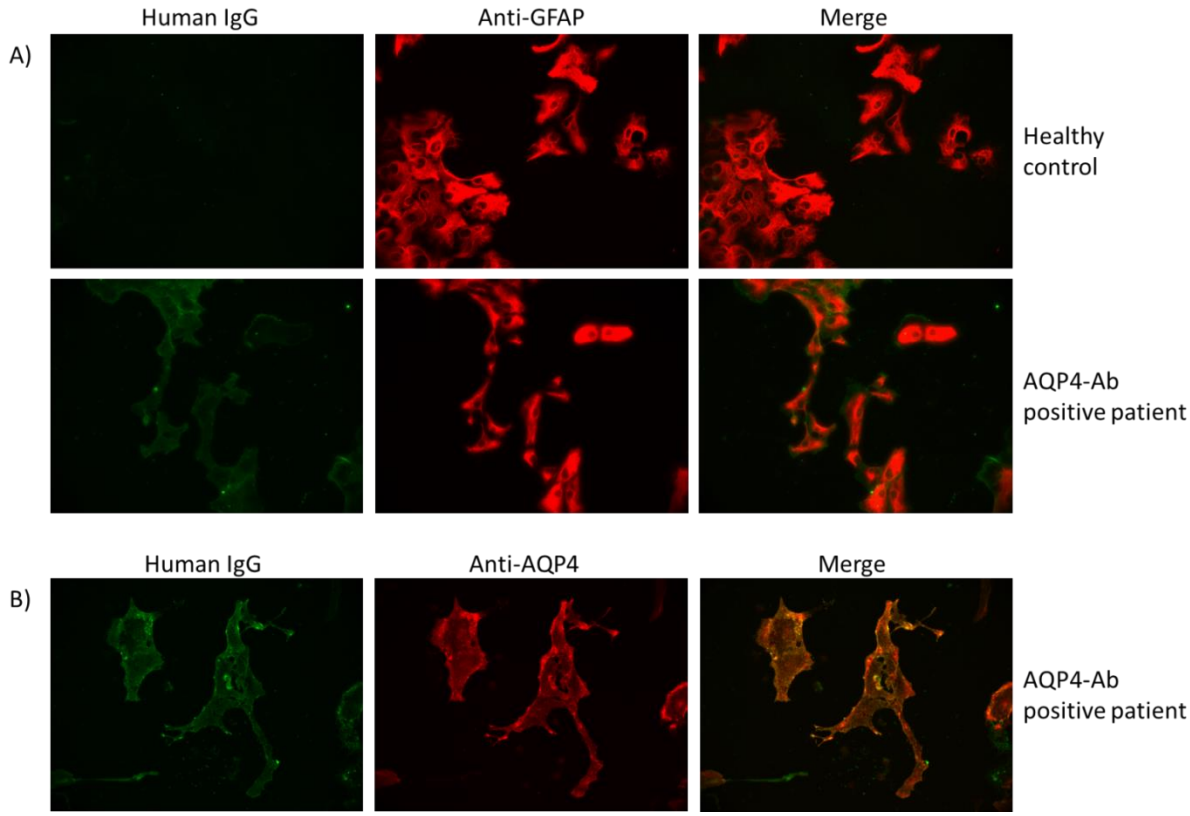


Figure 6.4. Astrocyte binding by human IgG in AQP4-Ab positive sera. A) IgG in AQP4-Ab positive patient sera binds to astrocytes established from P0 neonatal rat cortices and cultured for six days but healthy control sera does not. Binding of patient sera was detected using green fluorescent anti-human IgG. Astrocytes were visualised with red fluorescent anti-rabbit IgG after incubation with a rabbit polyclonal anti-GFAP antibody. Binding of human IgG extends beyond the margins of GFAP staining, probably because GFAP is an intracellular cytoskeletal protein, whereas human IgG should bind to AQP4 at the cell surface membrane. B) Patient IgG colocalises with AQP4. AQP4 was detected using a commercial rabbit polyclonal anti-AQP4 antibody, visualised with a red fluorescent anti-rabbit antibody.

or four hours also failed to increase sensitivity. However, overnight incubation at 4°C increased the sensitivity of the assay [30 of 37 (81%) AQP4-Ab positive patients demonstrated binding] and resulted in more intense staining (Figure 6.5). Additionally, a more punctate staining pattern was noted, which may represent clustering of AQP4 by patient IgG following prolonged exposure at 4°C, conditions in which internalisation won't occur.

The mean FACS score of samples that bound to astrocytes was higher than that of those that did not, though this difference was not statistically significant (Figure 6.6A) and there was a weak correlation between the strength of astrocyte binding and the FACS score (mean from two experiments) of AQP4-Ab positive samples (Figure 6.6B).

6.3.4.2 Binding of seronegative NMO/NMOSD sera to astrocytes in vitro

Following optimisation of astrocyte immunostaining experiments using AQP4-Ab positive sera and control sera, sera from the seronegative NMO/NMOSD cohort were screened for binding to astrocytes. Positive and negative control samples were included in experiments, analyses were performed blinded to AQP4-Ab and clinical status, and experiments were repeated three times.

When sera from AQP4-Ab negative NMO/NMOSD patients were incubated overnight at 4°C with astrocytes that had been cultured for five or six days, some binding was seen with seven of 96 seronegative NMO/NMOSD patient samples (Figure 6.7; Table 6.2). However, in all cases this binding was less intense than that seen with AQP4-Ab binding and was often associated with noticeable background staining. Additionally, none of these samples bound with a pattern resembling AQP4-Ab positive sera; a more speckled appearance was seen (Figure 6.7). Nevertheless, for all seven patients, a similar binding pattern was noted in at least two of three separate experiments when samples were analysed blinded, and no staining was seen with any of 11 healthy control or 18 MG control samples. Sera from one MS patient also showed a similar pattern of binding (Figure 6.7).

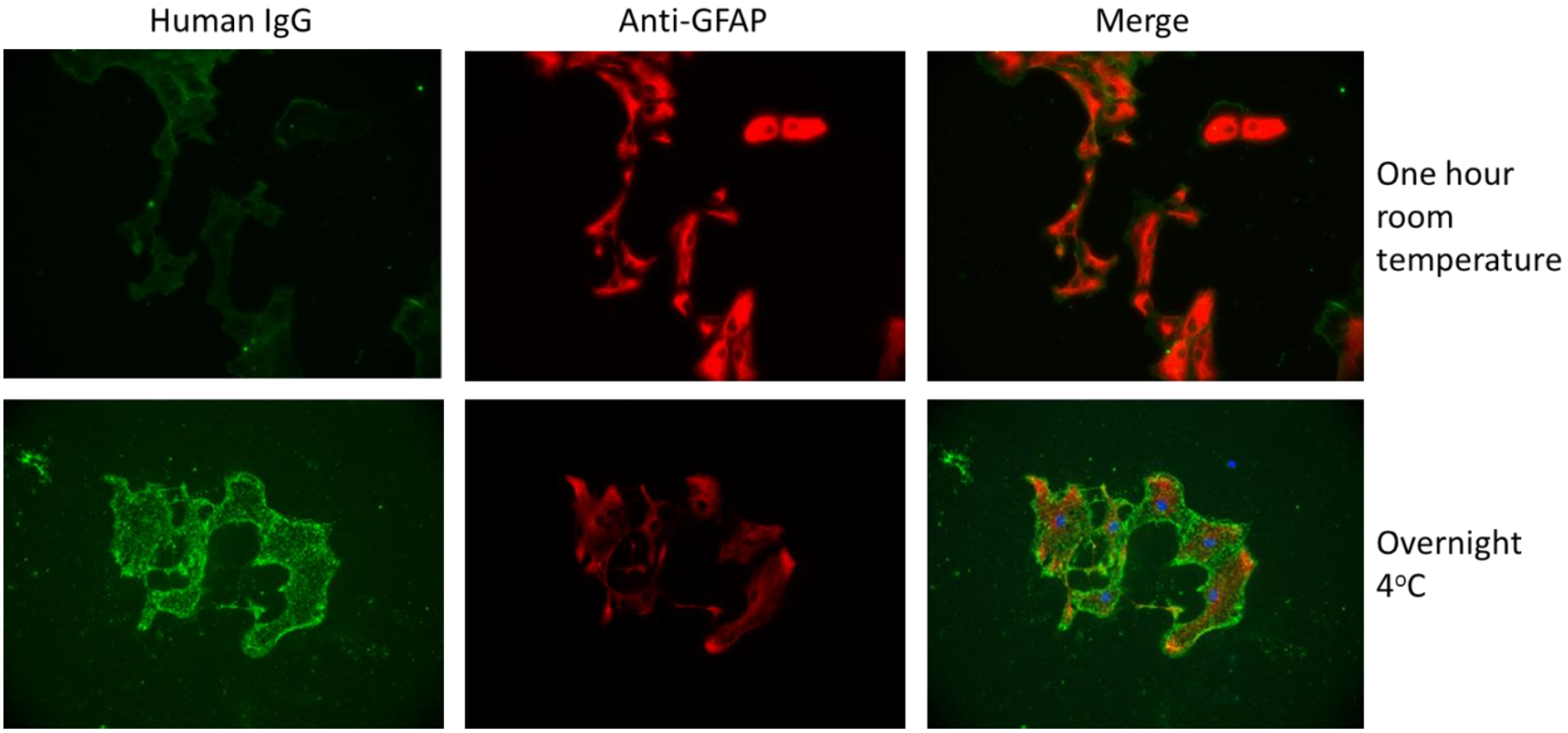


Figure 6.5. Comparison of overnight and one hour incubation of AQP4-Ab positive patient sera with astrocytes. Incubation of AQP4-Ab positive patient sera overnight at 4°C results in a more intense and punctate staining pattern of astrocytes than incubation for one hour at room temperature. Top panel shows AQP4-Ab positive patient sera (patient NMO 017; 1:100) incubated with day 6 P3 astrocytes for one hour at room temperature; bottom panel shows the same sample incubated with the same astrocytes overnight at 4°C.

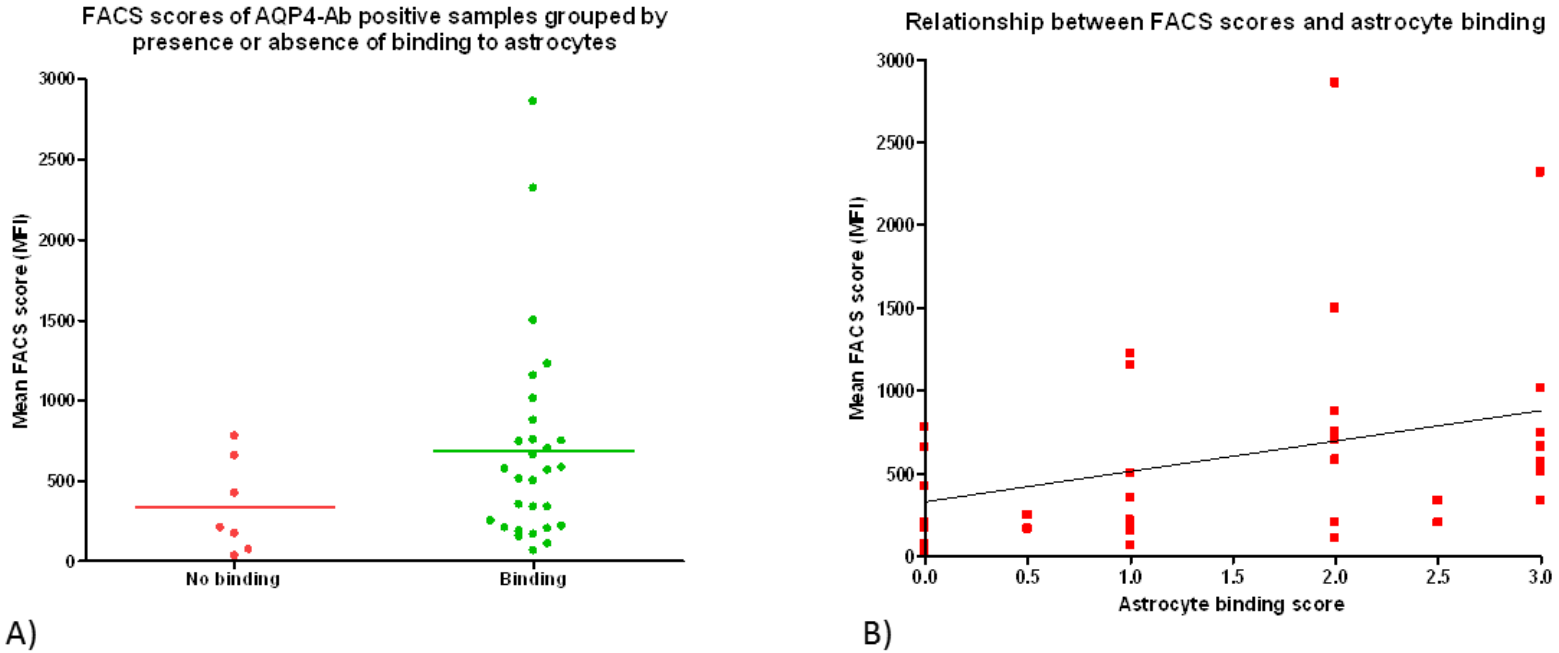


Figure 6.6. Relationship between FACS scores and astrocyte binding. A) The mean FACS score of AQP4-Ab positive serum samples that bound to astrocytes was higher than that of those that did not bind though this was not statistically significant (684.1 ± 642.8 vs. 333.5 ± 292.1; p=0.124). Binding to astrocytes was scored as 0 = no binding, 1= faint binding, 2 = moderate binding, 3 = strong binding. The mean binding score from two separate experiments was used. All samples were analysed blinded to AQP4-Ab status. B) There was a weak correlation between astrocyte binding score and FACS score (p=0.0472; r^2=0.111).

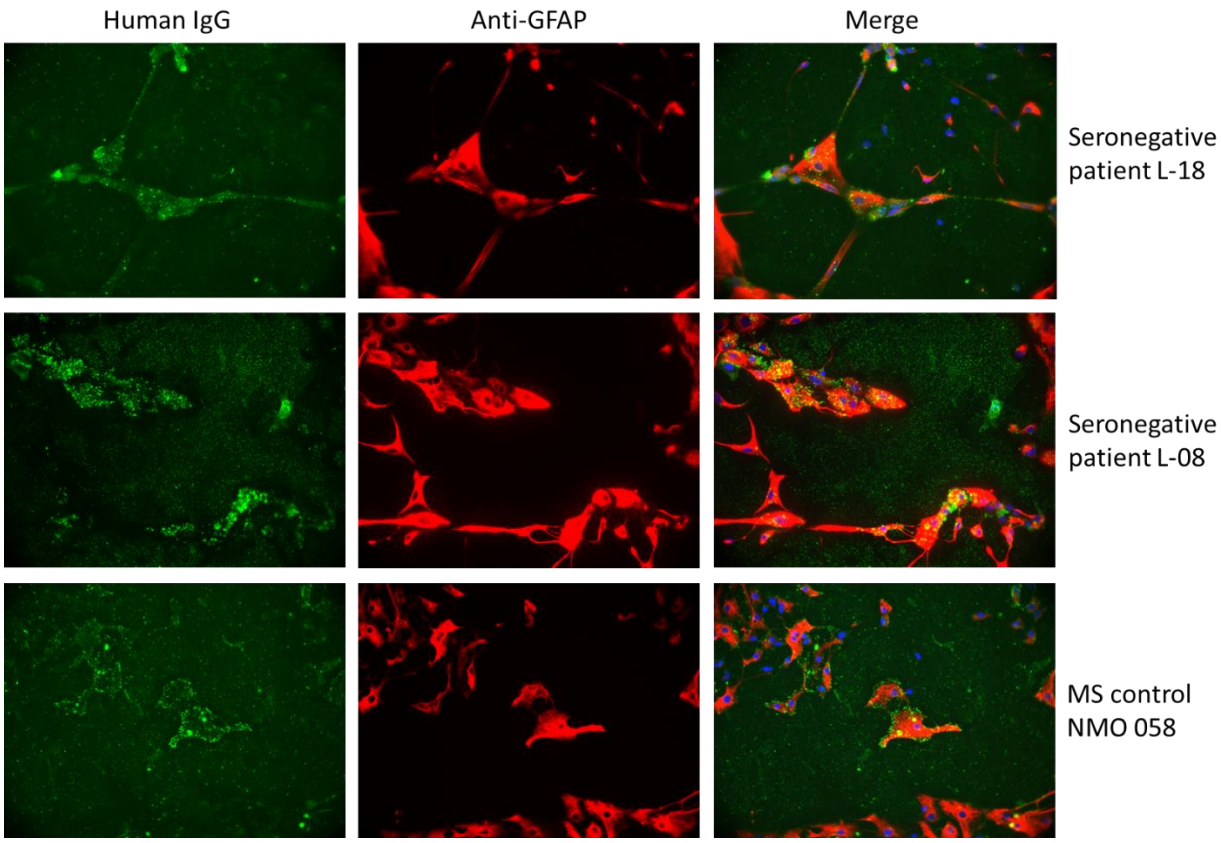


Figure 6.7. Examples of astrocyte binding by human IgG from seronegative NMO/NMOSD and MS patient sera. Human IgG from sera from several seronegative NMO patients bound to astrocytes with a speckled appearance (top and middle panels). Serum from a single MS control also bound to astrocytes (bottom panel). Binding of patient sera was detected with green fluorescent anti-human IgG. Astrocytes were visualised with GFAP staining. Right hand column shows merge. X40 magnification.

6.3.4.3 Non-astrocytic binding during astrocyte binding experiments

Whilst performing astrocyte immunostaining experiments, it was noted that several seronegative samples demonstrated speckled binding to cells other than astrocytes. This was suspected to be neuronal binding. Additionally, two samples from one patient (NMO 015) were noted to demonstrate binding in a pattern resembling oligodendrocytes.

6.3.4.4 Sera from a single seronegative NMO patient binds to oligodendrocytes in vitro

To confirm that the two samples from NMO 015 bound to oligodendrocytes, immunostaining experiments were repeated using mouse anti-2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase; an oligodendrocyte marker) to visualise oligodendrocytes. This confirmed that the patient IgG binding was to oligodendrocytes (Figure 6.8). This patient had relapsing ON and TM with a relatively short LETM at presentation (3 vertebral segments), but a very strong family history of MS and clinically was thought to have optico-spinal MS. MOG-Ab were negative.

6.3.4.5 Sera from some seronegative NMO patients bind to neurons in vitro

Because during astrocyte binding experiments, several seronegative NMO samples appeared to bind to neurons, the seronegative cohort and controls were systematically screened for neuronal binding. Optimisation of the neuronal binding protocol using NMDAR-Ab and contactin associated protein-2 (CASPR-2)-Ab positive sera revealed that neuronal immunostaining experiments were best performed with primary incubation at room temperature for one hour, and that the neurons required culturing for several days longer than astrocytes, and were therefore stained after seven to 10 days growth. Neurons were visualised with a mouse anti-microtubule-associated protein-2 (MAP-2; a neuronal marker) antibody.

Neuronal immunostaining revealed that multiple samples from two patients (NMO 035 and NMO 063) reproducibly bound to neurons in primary culture (Figure 6.9), whilst two other samples showed faint binding (Table 6.2).

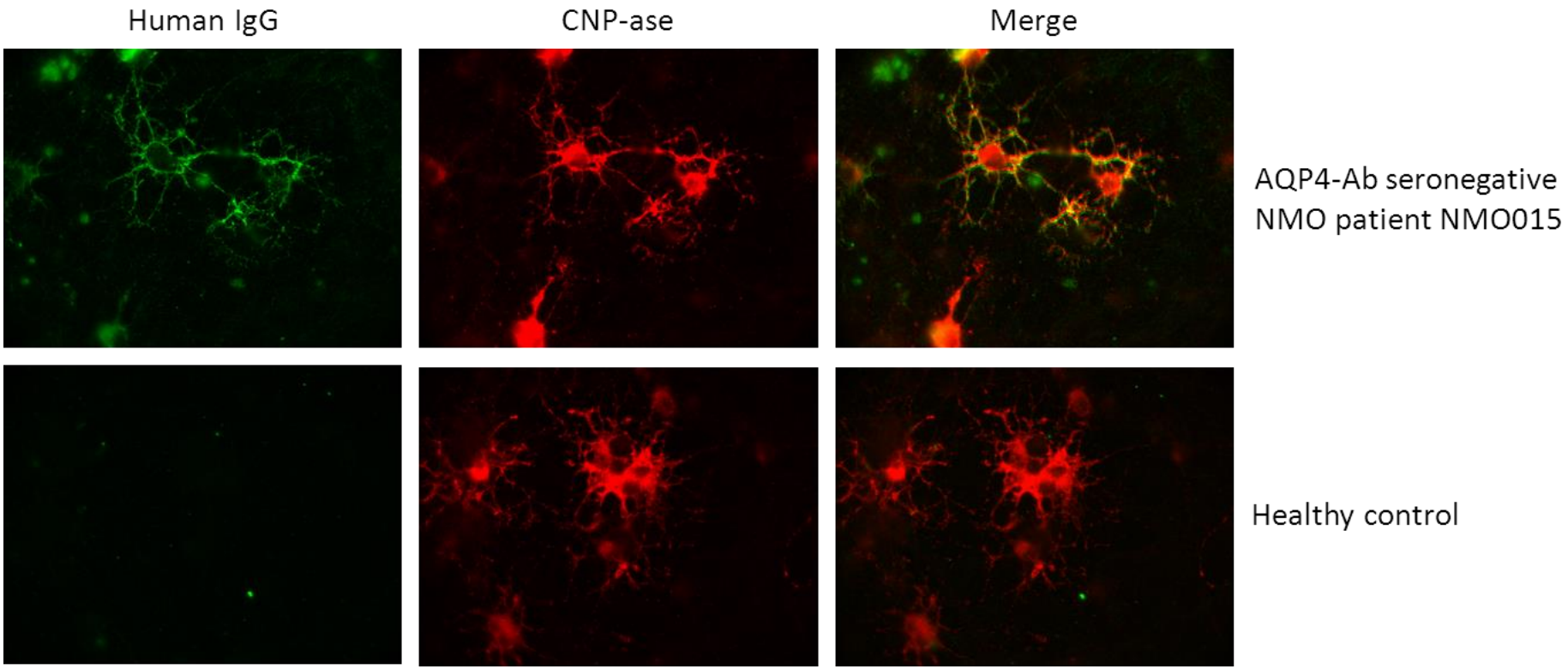


Figure 6.8. Oligodendrocyte binding by a seronegative NMO patient. IgG in sera from one seronegative patient bound to oligodendrocytes. Patient IgG is visualised with a green fluorescent anti-human antibody and oligodendrocytes are labelled with a mouse anti-CNPase antibody visualised with a red fluorescent anti-mouse antibody. Right hand column shows merge. Bottom row shows absence of oligodendrocyte binding with healthy control sera. X100 magnification.

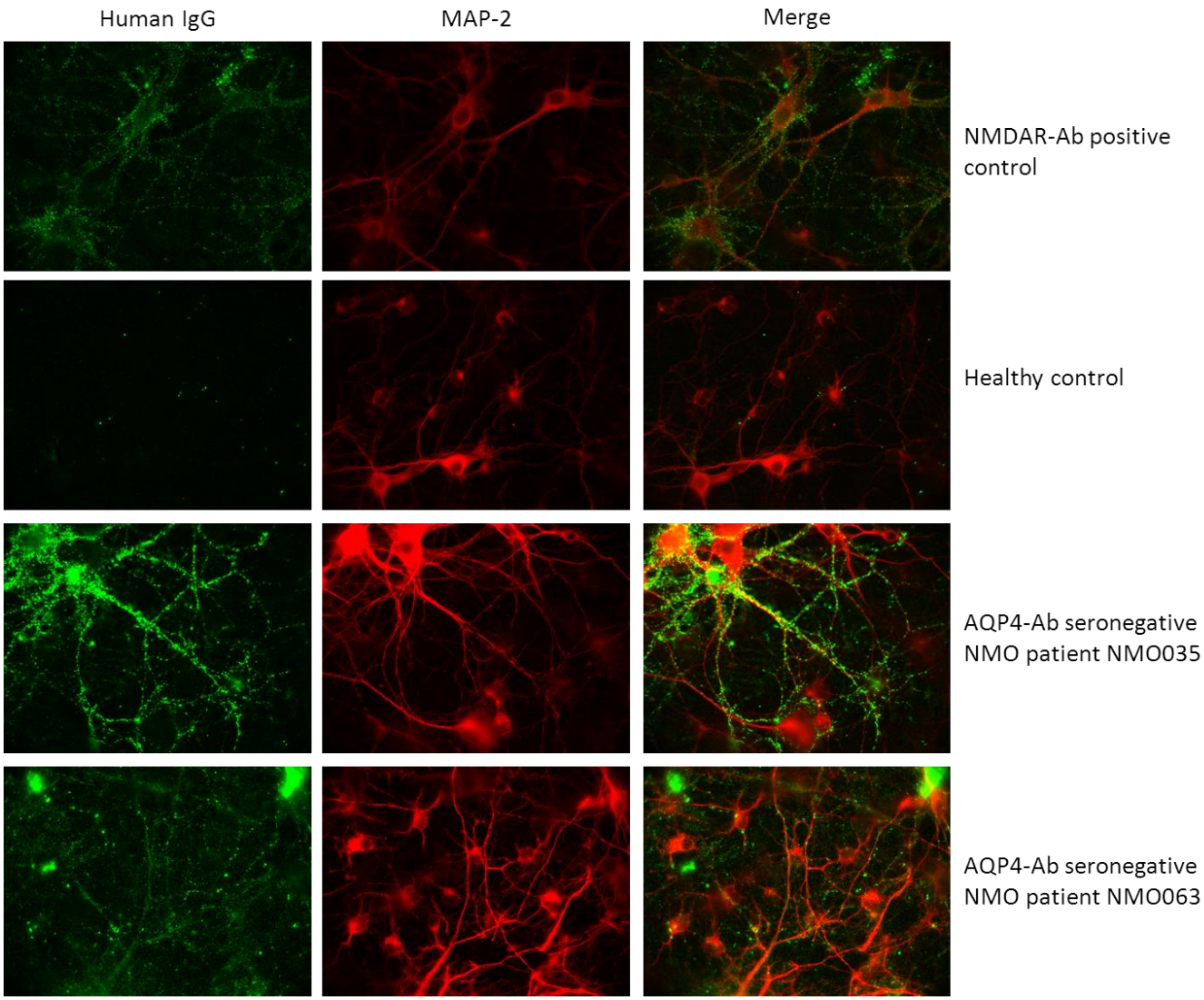


Figure 6.9. Binding of seronegative NMO/NMOSD patient sera to neurons in primary culture. Multiple samples from two seronegative NMO patients bound to neurons in primary culture (rows 3 and 4). Top row shows NMDAR-Ab positive control and second row shows healthy control for comparison. Patient IgG is visualised with a green fluorescent anti-human antibody and neurons are labelled with a mouse anti-MAP-2 antibody visualised with a red fluorescent anti-mouse antibody. Right hand column shows merge. X100 magnification.

Table 6.2. Summary of results of astrocyte and neuronal binding experiments

Group	**Samples demonstrating astrocyte binding (overnight incubation at 4ºC)**			**Samples demonstrating neuronal binding (1hour incubation at room temperature)**		
AQP4-Ab positive	30 of 37 (81%)			0 of 37 (0%)		
MS	1 of 25 (4%)			0 of 25 (0%)		
HC	0 of 11 (0%)			0 of 11 (0%)		
MG	0 of 18 (0%)			0 of 18 (0%)		
Seronegative NMO/NMOSD	7 of 96 (7%) (7 samples from 7 patients)			7 of 96 (7%) (7 samples from 4 patients)		
	Patient	Phenotype	Samples binding	Patient	Phenotype	Samples binding
	NMO 149	mLETM	1 of 1	NMO 063	rNMO	3 of 4
	NMO 034	rNMO	1 of 1	NMO 060	mNMO	1 of 2 (faint)
	L-18	rNMO	1 of 1	NMO 035	rNMO	2 of 3
	L-08	rLETM	1 of 1	NMO 130	mLETM	1 of 1 (faint)
	NMO 138	mNMO	1 of 1			
	NMO 143	mLETM	1 of 1			
	NMO 083	mON	1 of 1			

AQP4-Ab = aquaporin-4 antibody; MS = multiple sclerosis; HC = healthy control; MG = myasthenia gravis; NMO = neuromyelitis optica; NMOSD = neuromyelitis optica spectrum disorder; LETM = longitudinally extensive transverse myelitis; r = relapsing; m = monophasic; ON = optic neuritis.

6.3.4.6 Screening for other known neuronal antigens

Serum samples that showed definite or possible binding to cells in primary culture experiments were screened for binding to neuronal antigens available in the laboratory using CBAs. These included NMDAR, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptor (AMPAR), gamma-aminobutyric acid B receptor ($GABA_BR$), leucine-rich glioma inactivated 1 (LGI1) and CASPR-2. All samples were negative (score of 0) for all of these antigens.

6.3.5 Immunoprecipitation to identify novel antigenic targets

Having shown that some seronegative NMO/NMOSD patient sera bound to neurons, astrocytes or oligodendrocytes in primary culture, it was important to try and ascertain the antigenic targets. This was performed by immunoprecipitation, in which immune complexes between neuronal or glial cell antigens and human IgG were isolated through incubation with protein G sepharose beads and then eluted and visualised by electrophoresis and immunoblotting.

6.3.5.1 AQP4-Ab positive patient sera immunoprecipitates AQP4 from AQP4-expressing cell-lines

To ensure that this method could be used for identifying target antigens, it was first established that a known antigen could be immunoprecipitated by patient sera, and readily visualised. Thus, serum from a patient known to be strongly positive for AQP4-Ab was used for initial experiments. Serum at 1:20 dilution was incubated with HEK293 cells stably expressing the M23-AQP4 isoform to allow binding of patient AQP4-Ab to AQP4 at the cell surface. Cells were washed to remove unbound IgG, solubilised, and incubated with protein G sepharose beads which have high affinity for human IgG, thus capturing patient AQP4-Ab bound to M23-AQP4. The antibody-antigen complex was then eluted from the beads by heating to enable subsequent visualisation. To do this, the eluent was electrophoresed, immunoblotted and the immunoprecipitated AQP4 detected by incubation with a commercial polyclonal rabbit anti-AQP4 antibody, followed by incubation with an HRP-conjugated anti-rabbit antibody, visualised with ECL.

When M23-AQP4 expressing cells were incubated with high titre AQP4-Ab positive patient sera, several bands were seen on immunoblotting (Figure 6.10A). The largest of these were at approximately 45 kDa and 60 kDa. Further less distinct bands of higher molecular mass were also visualised. Although these bands were larger than the anticipated AQP4 band (32 kDa), none of these bands were seen in control samples, which included M23-AQP4 expressing cells incubated with healthy control sera, M23-AQP4 expressing cells incubated with no sera and untransfected HEK293 cells incubated with AQP4-Ab positive patient sera (Figure 6.10A). The experiment was repeated using three different AQP4-Ab positive samples and three different healthy control samples; identical bands were seen in all three AQP4-Ab positive patients but in none of the controls, strongly suggesting that the precipitated bands all represented AQP4 (Figure 6.10B).

Bands precipitated from cells transfected with the full length M1-AQP4 isoform were of slightly larger molecular weight than bands precipitated from cells expressing the shorter length M23-AQP4 isoform (Figure 6.11A).

AQP4-Ab positive sera incubated with astrocytes precipitated similar bands to those precipitated from M23-transfected cells using the same sera (Figure 6.11B). The bands precipitated from astrocytes were slightly wider and less distinct than those precipitated from M23-transfected cells and may represent precipitation of both M23 and M1 isoforms. No bands were precipitated from astrocytes using control sera.

To ensure that the protein bands weren't simply components of immunoglobulin from patient sera, serum from a strongly AQP4-Ab positive patient (NMO 017) was diluted 1:100 in LDS sample buffer, heated and electrophoresed. No bands were visualised after subsequent immunoblotting using the commercial AQP4-Ab, although protein bands were seen following Coomassie Blue staining, confirming that the sample had been successfully electrophoresed. Moreover, sera from a MOG-Ab positive patient also showed no bands, demonstrating that the visualised bands seen with AQP4-Ab positive samples were not merely a reflection of high serum IgG due to an autoantibody disease.

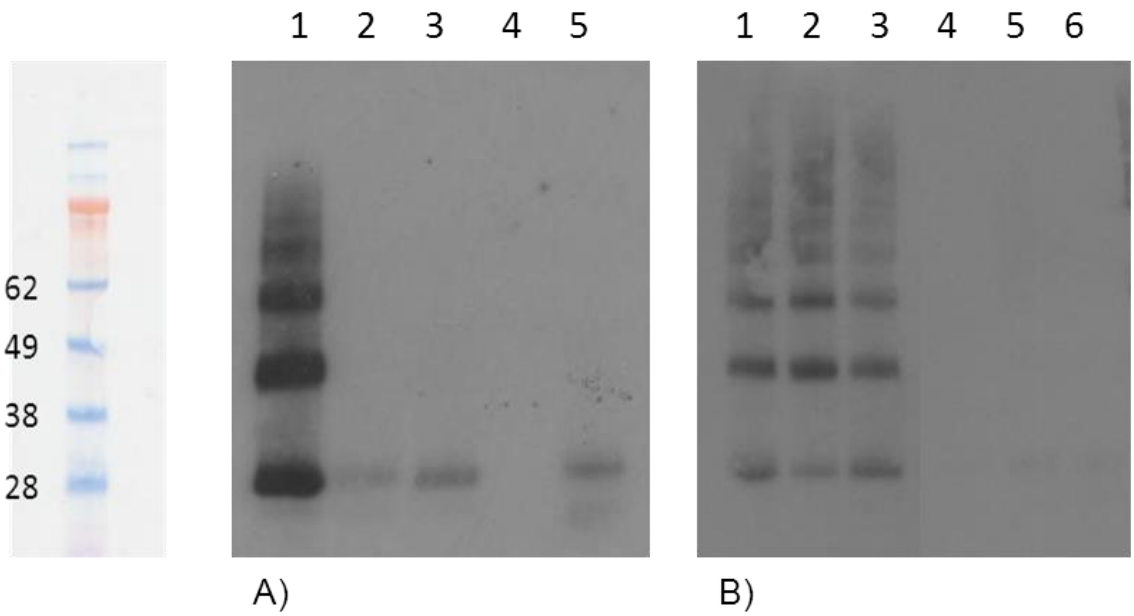


Figure 6.10. Immunoprecipitation of AQP4 from AQP4-expressing cell-lines by AQP4-Ab positive sera. A) AQP4-Ab positive patient sera immunoprecipitated proteins of approximately 45 kDa, 60 kDa and further larger bands when incubated with M23-AQP4 expressing cells (lane 1), but not when incubated with untransfected HEK cells (lane 5). Healthy control sera incubated with M23-AQP4 expressing cells did not immunoprecipitate these proteins (lane 2). Lane 1 = M23-AQP4 cells incubated with AQP4-Ab positive patient sera (NMO 017 1:20), lane 2 = M23-AQP4 cells incubated with healthy control sera (NMO 110 1:20), lane 3 = M23-AQP4 cells incubated with no sera, lane 4 = empty, lane 5 = untransfected HEK cells incubated with AQP4-Ab positive patient sera (NMO 017 1:20). The band at 25 kDa seen in all lanes likely represents cross-reactivity of the secondary antibody against IgG light chains (from patient sera and FCS used in buffers) and was reduced by changing the secondary antibody for later experiments. B) Sera from three different AQP4-Ab positive patients precipitated identical bands (lanes 1-3) whereas sera from three different healthy controls precipitated no bands when incubated with M23-AQP4 transfected HEK cells (lanes 4-6).

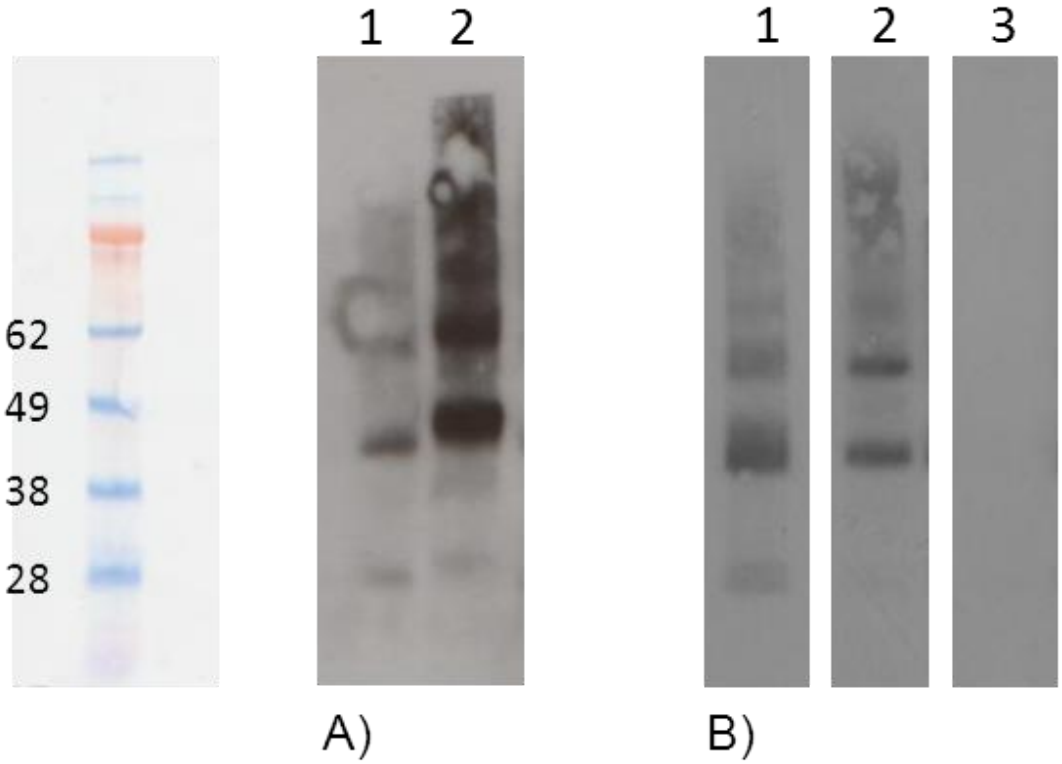


Figure 6.11. Comparison of immunoprecipitation from M23 and M1 transfected cells and from astrocytes. A) Bands precipitated from M23-transfected cells by AQP4-Ab patient sera were of slightly lower molecular weight (lane 1) than bands precipitated from M1-transfected cells (lane 2) using the same serum sample (NMO 017). B) AQP4-Ab positive patient sera (NMO 017) precipitated proteins of approximately 45 kDa and 60 kDa and larger less distinct bands from astrocytes in primary culture (lane 1). The bands seen were similar to those precipitated from M23-AQP4 transfected cells incubated with the same sera (lane 2), but were slightly wider and perhaps represented co-precipitation of both M23 and M1. No bands were precipitated from astrocytes by healthy control sera (lane 3).

Pre-adsorption of AQP4-Ab positive sera on M23-transfected cells was attempted but at the dilution required for pre-adsorption to be effective precipitated bands were too faint for the effect of pre-adsorption to be assessed.

6.3.5.2 Visualisation of unknown proteins on immunoblotting

It was shown above that AQP4 can be precipitated from both AQP4-transfected cells and from astrocytes in primary cultures using AQP4-Ab positive patient sera. However, in these experiments the precipitated AQP4 was detected using a commercial AQP4-Ab visualised with ECL, which is the most sensitive technique for visualising electroblotted proteins. However, to visualise unknown proteins, a commercial antibody against a specific target protein could obviously not be used. Therefore, protein visualisation by alternative methods was necessary.

6.3.5.2.1 Coomassie blue staining to detect immunoprecipitated proteins

One method of visualising unknown precipitated proteins is to use a commercial dye that is unselective in which proteins it binds to. Because following immunocapture and immunoprecipitation, the predominant proteins that should be in the eluent are the antigen of interest and human IgG, this method could be a suitable visualisation technique. The commercial Coomassie blue dye that was used (EZBlue, Sigma) can detect as little as 5 ng of protein in a gel (compared to 5 pg for Amersham ECL detection reagent). However, despite this, Coomassie blue staining of gels loaded with eluent precipitated from AQP4-transfected cells and astrocytes incubated with AQP4-Ab positive sera revealed no bands specific to AQP4-Ab positive sera (Figure 6.12A&B). This was despite using up to four 175 cm^2 flasks of cells per sample and despite showing that immunoblotting and ECL visualisation of a duplicate gel incubated with commercial AQP4-Ab revealed the previously noted bands, proving that the experiment had worked. Therefore, since this generic protein visualisation method could not even detect AQP4 precipitated with high titre AQP4-Ab positive sera, it seemed futile attempting similar experiments using the seronegative patient sera that bound to neurons, astrocytes or oligodendrocytes with less intensity than AQP4-Ab positive sera. The only exception was serum from seronegative patient NMO 035, which bound very strongly to neurons in primary culture. Immunoprecipitation

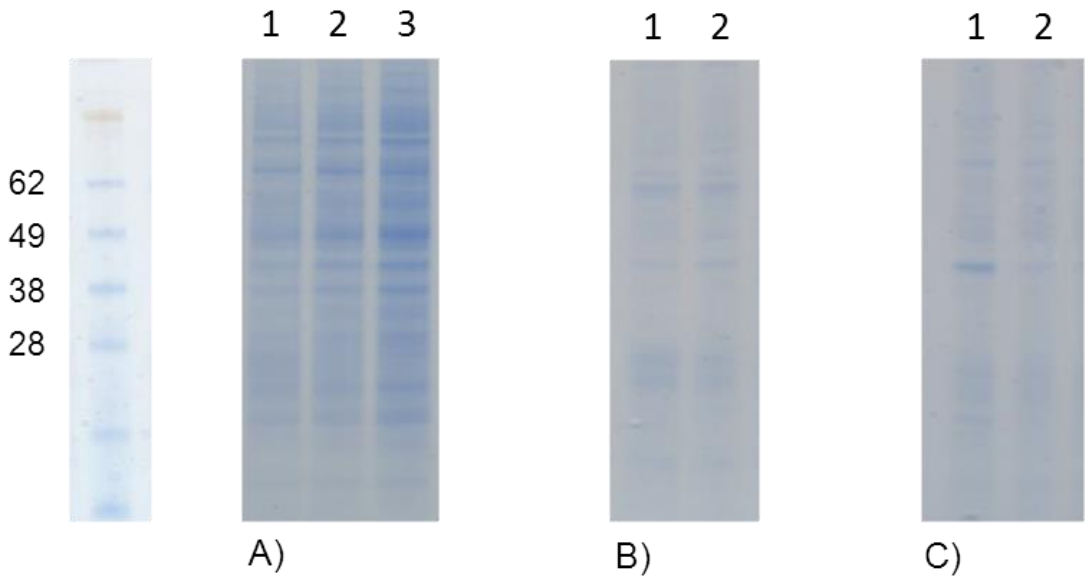


Figure 6.12. Coomassie Blue staining to visualise immunoprecipitated proteins. Immunoprecipitation of AQP4-transfected HEK cells (A), astrocytes (B) and neurons (C) incubated with AQP4-Ab positive and negative patient sera. A) Coomassie Blue staining of electrophoresed eluent from M23-transfected cells (lane 1) or M1-transfected cells (lane 2) incubated with AQP4-Ab positive patient sera failed to show any unique bands when compared to eluent from cells incubated with healthy control sera (lane 3). B) Staining of eluent from astrocyte cultures incubated overnight at 4^{o}C with AQP4-Ab patient sera (lane 1) did not show any unique bands compared with healthy control sera (lane 2). C) Eluent from neuronal cultures incubated for one hour at room temperature with sera from seronegative patient NMO 035 (lane 2) failed to identify any unique bands when compared to healthy control sera (lane 1).

experiments were attempted using this sera incubated with neurons but once again no unique bands were seen (Figure 6.12C).

6.3.5.2.2 Biotinylating cell surface proteins prior to immunoprecipitation

A second way of visualising unknown proteins is to biotinylate cell surface proteins, and thus the antigen of interest, prior to incubation with patient sera so that any membrane antigens bound by patient IgG are also conjugated to biotin and can thus be detected using a commercial HRP-conjugated streptavidin antibody, which has high affinity for biotin. When M23-transfected cells or astrocytes in primary culture were incubated with AQP4-Ab positive patient sera, no bands could be detected on subsequent immunoprecipitation and immunoblotting using streptavidin-HRP to detect biotinylated AQP4, and ECL as the method of visualisation. Incubating the same blot with a commercial AQP4-Ab demonstrated the bands seen in previous experiments proving that biotinylation did not prevent IgG binding or immunoprecipitation.

6.3.6 Immunoprecipitation of AQP4 using AQP4-Ab low positive samples

Since ECL visualisation techniques can detect minute quantities of a protein, it was investigated whether any of the seronegative NMO/NMOSD samples found to be low positive for AQP4-Ab on FACS could immunoprecipitate AQP4 from transfected cell-lines. One of these samples (NMO 128) was shown to immunoprecipitate the same bands as a strongly AQP4-Ab positive sample, whilst no bands were seen with the other two patient samples or with samples from AQP4-Ab positive patients who had converted to a negative serostatus on treatment.

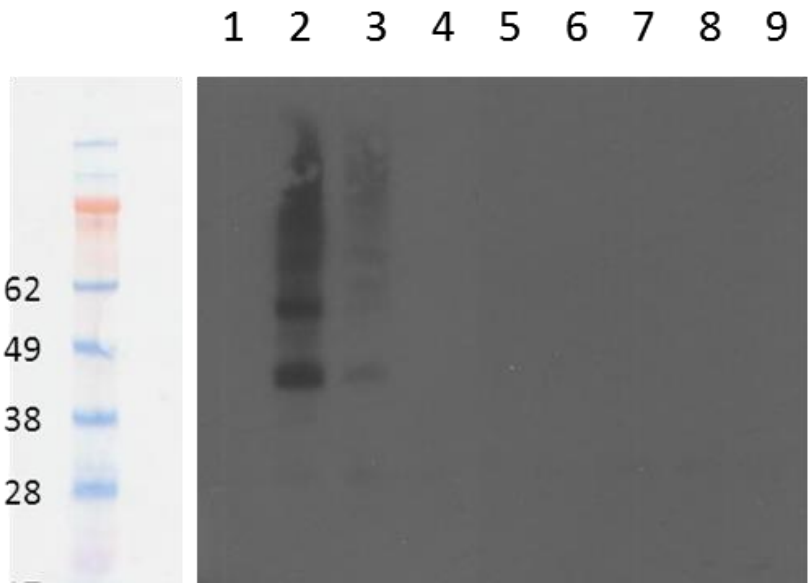


Figure 6.13. Immunoprecipitation of AQP4 by samples found to be low positive for AQP4-Ab on FACS. One of three seronegative NMO samples found to be low positive on FACS (NMO 128) was able to immunoprecipitate AQP4 from M23-AQP4 cells (lane 3), whilst samples from the other two patients did not (lanes 4 and 9). Lane 1 shows healthy control and lane 2 shows AQP4-Ab positive patient for comparison. None of four samples from AQP4-Ab positive patients who seroconverted to negative on treatment were able to immunoprecipitate AQP4 (lanes 6-8).

6.4 Discussion

It was shown in chapter 5 that NMO/NMOSD patients without AQP4-Ab show some important clinical and paraclinical differences when compared to NMO/NMOSD patients with AQP4-Ab. Some of these patients have alternative diagnoses, but a small number of patients exist who appear to have 'true' seronegative NMO/NMOSD. Despite much interest in seronegative NMO, very little work has been done looking at potential novel antigen targets. There have been no large studies to date investigating the behaviour of AQP4-Ab negative NMO/NMOSD patient sera *in vitro* and no large studies systematically looking for the presence of known or unknown antigens. In this chapter, we have shown that seronegative NMO/NMOSD likely represents a heterogeneous entity, comprising patients with low titre AQP4-Ab, patients with antibodies against non-AQP4 astrocytic antigens and patients with antibodies against neuronal and oligodendrocyte antigens together with patients who probably don't have an autoantibody-mediated condition.

6.4.1 Low levels of AQP4-Ab in seronegative NMO/NMOSD patients

When measured longitudinally over time, AQP4-Ab can disappear with treatment although they remain positive in the majority (Weinstock-Guttman, Miller et al, 2008; Kim, Lee et al, 2012). Conversely, patients who are seronegative to start with may become positive over time or during periods of disease activity (Weinstock-Guttman, Miller et al, 2008; Kim, Lee et al, 2012; Leite, Coutinho et al, 2012). Thus, it remains possible that some patients have very low levels of AQP4-Ab in their sera, which cannot be detected using current assays. Using the most sensitive AQP4-Ab assay available (i.e. FACS), three AQP4-Ab negative NMO/NMOSD patients tested borderline positive. Although increasing the concentration and volume of sera did not increase FACS scores, one of these patients tested positive on CBA at a 1:5 dilution and was able to immunoprecipitate AQP4 from AQP4-expressing cells. Thus, this patient was thought to be genuinely AQP4-Ab positive. The other two patients who tested low positive had multiple samples available for testing. One patient tested positive on only one sample and the other patient tested positive on both available samples. Neither demonstrated binding to astrocytes in primary culture but this does not exclude the possibility that they harboured low levels of AQP4-Ab. One MG and one MS control

also tested borderline positive and thus it may be that a low positive result in the seronegative NMO samples was not relevant. However, MG and NMO can co-exist (Leite, Coutinho et al, 2012) and NMO may be misdiagnosed as MS, particularly when mild and thus the possibility that the low positive results in these disease controls were relevant cannot be excluded. Further future improvements in AQP4-Ab assays will likely yield more low positive patients.

6.4.2 MOG-Ab in seronegative NMO/NMOSD patients

As mentioned in previous chapters, MOG is a potential candidate antigen in seronegative NMO because of its extracellular location, the fact that immunisation with MOG peptides can produce an NMO phenotype in animals and the recent reports of MOG-Ab in paediatric demyelinating disorders. Thus, we investigated whether MOG-Ab may be found in seronegative NMO/NMOSD patients. Six of the 63 (9.5%) seronegative NMO/NMOSD patients were positive for MOG-Ab. Three of these patients had a monophasic NMO phenotype and two had monophasic LETM. One patient, followed at the Walton Centre, Liverpool apparently has a relapsing NMO phenotype though relapses were mild and not radiologically proven. The clinical features of the MOG-Ab positive patients seen within the Oxford NMO clinical service will be discussed in detail in chapter seven.

There are several lines of evidence to suggest that MOG-Ab have pathogenic potential. Firstly, immunisation of rodents with MOG peptides produces EAE. Secondly, in fetal rat brain cultures, incubation with a monoclonal MOG-Ab and complement results in demyelination (Kerlero de Rosbo et al 1990). Thirdly, MOG-Ab have been shown to be predominantly of the IgG1 subtype (McLaughlin, Chitnis et al, 2009; Mader, Gredler et al, 2011; Probstel, Dornmair et al, 2011), a subtype that can fix complement, and sera from MOG-Ab-positive patients can cause death of MOG-transfected cells *in vitro* (Zhou, Srivastava et al, 2006; Brilot, Dale et al, 2009) via complement mediated cytotoxicity (Zhou, Srivastava et al, 2006) or by ADCC in the presence of NK cells (Brilot, Dale et al, 2009). Fourthly, sera from MOG-Ab positive patients has been shown to bind to myelin in rat brain sections (Zhou, Srivastava et al, 2006) and to normal human white matter in pathological specimens (McLaughlin, Chitnis et al, 2009). Finally, Dr Mark Woodhall

in the Oxford Neuro-immunology laboratory has shown that when measured longitudinally, MOG-Ab titres decrease over time following clinical recovery, which would be consistent with a pathogenic role.

We have found MOG-Ab in a significant proportion of seronegative NMO/NMOSD patients. These antibodies appear to have pathogenic potential and may therefore be an important disease biomarker in seronegative NMO. Further studies in independent cohorts are now required to confirm our findings.

6.4.3 GlyR-Ab in seronegative NMO/NMOSD patients

Glycine, together with GABA, is an important inhibitory neurotransmitter that exerts its effects via the GlyR, which belongs to the Cys-loop family of ligand-gated ion channel receptors. Five GlyR subunits exist – four α subunits (α1-α4) and a single β subunit and the GlyR exists as a pentamer of subunits arranged around a central ion channel pore; α1 is the predominant subunit found in rodents. GlyR are expressed predominantly in the spinal cord, brainstem and retina (Araki, Yamano et al, 1988). GlyR-Ab were first identified in the syndrome of progressive encephalomyelitis, rigidity and myoclonus (PERM) (Hutchinson, Waters et al, 2008) when antibodies against the α1 subunit were detected using a CBA in a patient with this syndrome who responded clinically and immunologically to immunosuppressive treatment. GlyR-Ab have subsequently been confirmed in PERM in several small case series (Mas, Saiz et al, 2011; Shugaiv, Leite et al, 2013). Because of the anatomical location of GlyR and because GlyR-Ab have been found in the sera of some patients sent to the Oxford laboratory for AQP4-Ab testing, the GlyR is another possible candidate antigen in seronegative NMO and the seronegative NMO/NMOSD cohort were screened for GlyR-Ab. Only one seronegative NMO/NMOSD patient (with monophasic NMO) was GlyR-Ab positive, and only at low levels. Additionally, one AQP4-Ab positive and one MOG-Ab positive sample were also low positive for GlyR-Ab suggesting that GlyR-Ab, at least at low levels, may be non-specific or that GlyR-Ab may occur as a secondary phenomenon in patients with CNS inflammation. However, a recent Turkish study found GlyR-Ab in two of 10 seronegative NMO patients (Cavus, Ugurel et al, 2013). GlyR-Ab were tested using

the Oxford CBA and so methodological differences cannot explain why a much higher proportion of their seronegative NMO patients were GlyR-Ab positive. The possible role of GlyR-Ab in AQP4-Ab negative NMO/NMOSD warrants further evaluation and clinical details on the patients sent for AQP4-Ab testing but who have GlyR-Ab need to be obtained.

6.4.4 The role of astrocytes in seronegative NMO/NMOSD

AQP4 is found predominantly on astrocytes and there is now substantial evidence to suggest that AQP4-Ab disease is an astrocytopathy. IgG in sera from AQP4-Ab positive NMO/NMOSD patients binds to AQP4 on astrocytes in primary culture (Hinson, Roemer et al, 2008; Vincent, Saikali et al, 2008; Sabater, Giralt et al, 2009; Marignier, Nicolle et al, 2010), and when injected into rodents with established CNS inflammation leads to perivascular loss of AQP4 and GFAP (Bradl, Misu et al, 2009; Kinoshita, Nakatsuji, et al 2009b; Kinoshita, Nakatsuji, et al, 2010; Saadoun, Waters et al, 2010; Sharma, Fischer et al, 2010) and astrocyte death (Bennett, Lam et al 2009; Bradl, Misu et al, 2009; Kinoshita, Nakatsuji, et al 2009b; Kinoshita, Nakatsuji, et al, 2010; Saadoun, Waters et al, 2010). Additionally, biomarkers of astrocyte damage, such as GFAP, are found in the CSF of NMO patients (Takano, Misu et al, 2008; Misu, Takano et al, 2009; Takano, Misu et al, 2010; Petzold, Marignier et al, 2011) and neuropathological specimens from NMO patients show astrocyte loss within lesions (Misu, Fujihara et al, 2006; Misu, Fujihara et al, 2007; Roemer, Parisi et al, 2007; Yanagawa, Kawachi, et al 2009; Lee, Metz et al, 2010).

There has been relatively little work looking at the behaviour of seronegative NMO/NMOSD patient sera *in vitro* and *in vivo* and it is not known whether these patients also have an astrocytopathy. There have been no neuropathological studies in seronegative NMO patients. One study has shown that CSF GFAP in a small number of seronegative NMO/NMOSD patients was elevated to a similar degree as seropositive patients (Petzold, Marignier et al, 2010) but seronegativity was defined using the insensitive tissue IIF assay and thus these patients may have been false negatives. Experimental studies using seronegative patient sera are also scarce. One study showed that pooled sera from 16 seronegative NMO patients could cause astrocyte death *in vitro* (Sabater, Giralt et al, 2009), but again, this study defined seropositivity using tissue IIF and

may have included false negatives. Another study, which also defined AQP4-Ab seropositivity using this insensitive assay showed that whilst seropositive sera led to astrocyte and oligodendrocyte death *in vitro*, IgG from two seronegative patients did not (Marignier, Nicolle et al, 2010). In animal models, injection of rats with EAE with human IgG from two seronegative NMO patients failed to produce the NMO-like lesions seen following injection with AQP4-Ab positive IgG (Bradl, Misu et al, 2009). This is the only study to have defined seropositivity using CBA. Thus, overall, there is insufficient evidence to confirm or refute the possibility that seronegative NMO/NMOSD is also an astrocytopathy.

In the current study, the majority of AQP4-Ab positive NMO/NMOSD serum samples bound to astrocytes *in vitro*, compared to only a minority of sera from seronegative NMO/NMOSD patients. Since sera from some AQP4-Ab positive NMO/NMOSD patients did not bind, particularly those with lower titre AQP4-Ab, this does not rule out the possibility that some seronegative NMO patients have low levels of AQP4-Ab. However, it makes it unlikely that seronegative NMO/NMOSD patients have antibodies against an alternative astrocytic antigen. The few seronegative samples that did bind to astrocytes did so only weakly and often in association with a significant amount of background staining, suggesting that the observed binding may have been non-specific. However, no similar binding was noted in any healthy or MG control samples and in only one MS control sample, and thus it may be significant. The AQP4-Ab negative patients whose sera bound to astrocytes had mixed clinical diagnoses. One patient had 'true' relapsing seronegative NMO and one had idiopathic relapsing LETM, whilst another had idiopathic monophasic NMO and three had CNS inflammation in the context of infection (one TB, one Epstein Barr virus, one post varicella-zoster infection). This makes is less likely that a single astrocytic antigen was the target for IgG in these samples, and suggests that astrocyte binding may be non-specific.

Further work is needed to establish the potential significance of this weak astrocytic binding in some seronegative samples. It would have been interesting to quantify CSF biomarkers of astrocyte damage in these patients but unfortunately no CSF samples were available. Complement

activation and astrocyte viability experiments to see whether these sera can activate complement on astrocytic membranes and damage or kill astrocytes would also have been helpful in establishing potential astrocyte pathogenicity in these samples.

6.4.5 Antibodies to neuronal antigens in seronegative NMO/NMOSD

Although there have been isolated reports of seronegative NMO/NMOSD patients testing positive for other antibodies such as NMDAR-Ab (Kruer, Koch et al, 2010; Pennington, Livingstone et al, 2012) and the paraneoplastic antibody CRMP5 (Jarius, Wandinger et al, 2012) there have been no large and systematic studies looking for the presence of these and other autoantibodies in seronegative NMO/NMOSD patients.

In this chapter, a large cohort of seronegative NMO/NMOSD patients were screened for IgG binding to neurons in primary culture. Multiple samples from two seronegative NMO patients bound to neurons. Both of these patients had 'true' seronegative NMO, which was relapsing and appeared to be responsive to immunosuppression. One of these patients (NMO 035) had undergone thymectomy for a thymoma. Many thymoma patients have autoantibodies in their sera and it may be that the neuronal binding wasn't relevant to the patient's NMO. However, three samples were available for this patient; all were taken several years after thymoma excision. The earliest sample did not bind to neurons, whilst the two most recent samples did. Although these samples were all taken during disease remission, the two that demonstrated IgG binding to neurons were taken following reduction of immunosuppression. This may suggest that the antibodies were being suppressed on treatment, but became detectable following dose reduction. For the other patient whose IgG bound to neurons (NMO 063), three samples were available; two were taken during relapses and one during a period of disease remission. Interestingly, neuronal binding was detected on the two relapse samples, but not with the sample taken during remission, suggesting that the IgG binding to the neurons may be of pathogenic relevance. In chapter 5 we showed that when a sensitive AQP4-Ab assay is used and other causes have been excluded, 'true' relapsing seronegative NMO is rare. It is interesting, therefore, that the two patients with neuronal IgG

binding both fell into this diagnostic group and it is possible that the neuronal binding may be relevant to the disease.

In an attempt to identify the neuronal target antigens, immunocapture and immunoprecipitation experiments were planned. Unfortunately, although AQP4 could be immunoprecipitated from AQP4-expressing cell-lines and from astrocytes in primary culture using AQP4-Ab positive sera, the precipitated protein could only be detected using a commercial AQP4-Ab and not using techniques suitable for detecting unknown proteins. When biotinylated M23-transfected cells or astrocytes were incubated in primary culture with AQP4-Ab positive patient sera, no bands could be detected on subsequent immunoprecipitation and immunoblotting using streptavidin-HRP and ECL to visualise biotinylated AQP4. Incubating the same blots with a commercial AQP4-Ab demonstrated bands, proving that biotinylation did not prevent binding of AQP4-Ab to AQP4 and that experiments had worked. The most likely reasons therefore for inability to visualise the proteins were either that the streptavidin-HRP commercial antibody was of insufficient sensitivity or that the amount of biotin bound to AQP4 was below the detection limit of the streptavidin-HRP commercial antibody. The extracellular portion of AQP4 is small and thus may not be amenable to biotinylation. Attempted visualisation of precipitated proteins using a commercial Coomassie Blue dye also failed to reveal the bands seen with ECL, probably because the amount of precipitated protein was below the detection limit of the technique. Immunoprecipitation was also attempted using samples from patient NMO 035, which bound strongly to neurons, but again no specific bands were seen. This may be because the antigen was bound with insufficient avidity for immunocapture. Alternatively, it may be because the amount of bound antigen was very small and therefore below the detection limit of the visualisation techniques, or because the molecular weight of the antigen fell outside the limits of the gels used for experiments.

Identifying novel antigens through immunocapture and immunoprecipitation can be difficult, and it can take years to refine assays in order to be successful. Some antigens may not be amenable to immunoprecipitation and our lack of success does not exclude the possibility that the patients reported here may have autoantibodies against neuronal antigens that are relevant in seronegative

NMO. Screening of independent seronegative NMO/NMOSD cohorts is needed to confirm whether neuronal antigens may be potential targets in seronegative NMO/NMOSD and further work is required to identify these antigens. If several patients are identified with common clinical features and neuronal binding *in vitro*, immunohistochemistry on murine CNS cryosections may reveal common staining patterns that can help direct investigators towards potential target antigens. Once these antigens are identified, CBAs or other assays that can concentrate the antigen may then identify more patients with antibodies targeting these proteins.

6.4.6 Antibodies to oligodendrocyte antigens in seronegative NMO/NMOSD

Although primary cultures in this work were not set up for oligodendrocyte growth and maturation, oligodendrocytes were seen within the cultures and two samples from one seronegative NMO patient were shown to bind to oligodendrocytes. Oligodendrocyte antigens are potential targets in seronegative NMO/NMOSD and it would be interesting to screen the cohort for binding to oligodendrocytes that have been cultured specifically to express myelin. Unfortunately, this facility was not available in the Oxford laboratory but future collaboration with laboratories that do perform such work may be possible.

6.4.7 Conclusions

It appears from the work in this chapter that seronegative patients are an aetiologically heterogeneous group. Some have low levels of AQP4-Ab and some have MOG or GlyR Abs. Others bind to astrocytes, neurons or oligodendrocytes in primary cultures. Further studies are required to characterise the role of MOG and GlyR Abs in seronegative NMO and to investigate novel astrocytic and neuronal antigen targets.

Chapter 7: Characterisation of NMO/NMOSD patients with myelin oligodendrocyte glycoprotein antibodies

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7.1 Introduction

MOG is a CNS-specific antigen that is expressed on the outer surface of myelin sheaths. Like AQP4, MOG is accessible to autoantibodies in the extracellular space and MOG-Ab are therefore potentially pathogenic. Although MOG-Ab have been described for many years in patients with MS, methodological problems with early assays make it difficult to interpret the results of many of these studies. It is only recently that assays suitable for detecting specific and potentially pathogenic antibodies have been established; using these assays, MOG-Ab have been found largely in paediatric demyelinating disorders (O'Connor, McLaughlin et al, 2007; Brilot, Dale et al; 2009; Selter, Brilot et al, 2010; Di Pauli, Mader et al, 2011; Lalive, Hausler, et al 2011). To date, only one study has screened a small cohort (n=13) of adult NMO patients, none of whom tested positive (McLaughlin, Chitnis et al, 2009). A MOG-Ab CBA has recently been developed in the Oxford laboratory by Drs Patrick Waters and Mark Woodhall. In chapter 6, screening of a large cohort of AQP4-Ab seronegative NMO/NMOSD patients revealed that a significant proportion had antibodies to MOG. Since the development of the Oxford MOG-Ab assay, patients seen within the Oxford NMO clinical service with a first demyelinating event have been routinely screened for both AQP4-Ab and MOG-Ab. Here, the features of patients seen within the Oxford NMO service with MOG-Ab are characterised and compared to those of patients with AQP4-Ab.

7.2 Methods

The Oxford NMO clinical database was searched for patients presenting with a first CNS inflammatory demyelinating event between January 2010 and April 2013 who tested positive for MOG-Ab or AQP4-Ab. Data have been prospectively entered into this database over this time period and clinical and paraclinical data were collated retrospectively. The features of MOG-Ab positive NMO/NMOSD patients were compared with those of AQP4-Ab positive patients.

7.3 Results

Nine MOG-Ab positive patients and 20 AQP4-Ab positive patients were identified. No patients tested positive for both antibodies.

7.3.1 Characterisation of MOG-Ab positive patients

7.3.1.1 Demographics of MOG-Ab positive patients

Of the nine patients testing positive for MOG-Ab, five were males and four were females. The majority of patients (n=7) were Caucasian and two were Asian. Two patients were children and seven were adults and the mean age at disease onset was 32.3 ± 17.1 years (range 3.9 to 58.3 years).

7.3.1.2 Clinical features of MOG-Ab positive patients

The clinical features of MOG-Ab patients are summarised in Table 7.1. All MOG-Ab positive patients presented acutely with CNS inflammation and all had myelitis at onset, with or without evidence of involvement of the brain or optic nerves. Four patients presented with simultaneous or rapidly sequential ON and LETM, three patients had isolated LETM, two had LETM and symptomatic brain lesions and one had myelitis with short cord lesions on MRI. Overall, attacks tended to be severe, with median nadir EDSS of 6.0. Where the eyes were affected, vision was markedly impaired and all four patients with ON had visual acuities of less than 6/60 in at least one eye. Three of the four patients with ON had clinical involvement of both eyes and overall, acuities were less than 6/60 in five of seven affected eyes. Optic disc swelling was a prominent feature,

being present in six of seven affected eyes (the presence or absence of disc swelling in the remaining affected eye was not documented).

In myelitis attacks, the bladder seemed to be affected early, with three patients presenting with sphincter disturbance prior to development of sensory or motor impairment. No patients had respiratory symptoms. Both children and one adult had mild encephalopathy. Eight of the nine patients were hospitalised but none required intensive care admission.

Table 7.1. Clinical features of MOG-Ab positive NMO/NMOSD patients

Pt	Sex/Age	Ethnicity	Clinical features	Other comorbidities	Treatment	FU (months)	Outcome
1	F 32	Asian	Severe unilateral optic neuritis; severe myelitis on reducing steroids	Thyroid carcinoma with thyroidectomy and radio-iodine	IVMP then oral steroids for 1 year; azathioprine for 2 years	39	Good recovery; some residual bladder symptoms
2	M 27	Asian	Severe myelitis	Usher's syndrome	IVMP and PEX then oral steroids for 2 years	37	Full recovery
3	M 34	Caucasian	Severe bilateral optic neuritis, mild myelitis	Nil	IVMP	25	Full recovery
4	M 32	Caucasian	Severe bilateral optic neuritis, severe myelitis	Nil	IVMP and PEX then oral steroids for 1 year	18	Full recovery
5	M 4	Caucasian	Severe bilateral optic neuritis, mild myelitis, encephalopathy	Nil	IVMP then oral steroids for 9 months	10	Full recovery
6	M 49	Caucasian	Severe myelitis, brainstem symptoms, encephalopathy	Nil	IVMP and PEX then oral steroids	4	Full recovery
7	F 58	Caucasian	Mild myelitis	Carcinoma of the breast	IVMP then oral steroids	4	Full recovery
8	F 41	Caucasian	Mild myelitis	Fibromyalgia, hypothyroidism	Oral methylprednisolone then oral prednisolone for 24 months	24	Good recovery; neurological examination normal but subjectively poly-symptomatic
9	F 11	Caucasian	Severe myelitis, encephalopathy	Nil	IVMP then oral steroids	5	Full recovery

Pt = patient; M = male; F = female; IVMP = intravenous methylprednisolone; PEX = plasma exchange; FU = follow up.

All patients received high dose corticosteroids in the acute setting and three additionally underwent plasma exchange. Remarkably, all patients made a dramatic and prompt improvement following treatment. Patient 1 was left with mild bladder symptoms and patient 8 was left with moderate bladder symptoms, and has severe fibromyalgia and walks with a stick though has a normal neurological examination. Otherwise, all patients made a full motor and visual recovery. No patients have experienced any further attacks over a median follow-up period of 18 months (range 2 to 39), though the three patients with the shortest follow-up duration remain on a tapering dose of oral steroids.

7.3.1.3 Radiological and other paraclinical features of MOG-Ab positive patients

The radiological and other paraclinical features of MOG-Ab positive patients are summarised in Table 7.2. All MOG-Ab positive patients had radiological evidence of spinal cord involvement in the acute setting. In eight of the nine patients, the spinal cord T2 hyperintensity was longitudinally extensive and five patients had multiple lesions (not all lesions were longitudinally extensive). Lesions often involved the central grey matter (five of six patients with adequate axial imaging), were associated with cord oedema in all cases, and hypointensity on T1 weighted imaging was present in the majority (five of eight patients with adequate imaging). There appeared to be a predilection for conus involvement, with six of eight patients (75%) with conus imaging demonstrating T2 hyperintensity in this region (Figure 7.1). Post contrast enhancement was relatively uncommon, being present in only two of six patients administered contrast. Eight patients had follow-up imaging performed at least three months after the acute attack and remarkably there was complete resolution of T2 high signal in six cases, with no evidence of cord atrophy. One patient with follow-up imaging at three months had mild residual signal abnormality and patient 8 who demonstrated several short cord lesions at onset probably still had several short cord lesions on follow-up imaging, though scan quality was poor.

Table 7.2. Paraclinical features of MOG-Ab positive NMO/NMOSD patients

Pt	CSF	Brain MRI		Spinal cord MRI	
		Acute	**FU (months)**	**Acute**	**FU (months)**
1	WBC 187/mm^3, protein 1.10 g/L; no OCBs	Single lesion left cerebellar peduncle	Normal (15)	T2 high signal T3 to conus with cord expansion but no post contrast enhancement	Normal (15)
2	WBC 111/mm^3, protein 0.83 g/L; matched OCBs	Single lesion adjacent to 4th ventricle	Normal (7)	T2 high signal C3 to C6 and T6 to conus with cord expansion and post contrast enhancement	Normal (7)
3	Normal	Multiple T2 lesions in cerebellum and thalamus; swelling of both optic nerves on CT	Normal (12)	T2 high signal C4 to 6 and T5/6 with mild cord expansion; contrast not given	Normal (12)
4	WBC 92/mm^3, protein 0.76 g/L; matched OCBs	Normal except T2 high signal both optic nerves	Normal (17)	T2 high signal T4 to conus with cord expansion but no post contrast enhancement	Normal (17)
5	Normal; no OCBs	Large fluffy lesions in left basal ganglia, and juxtacortical and subcortical lesions with contrast enhancement; enhancement of both optic nerves	Normal (6)	T2 high signal in conus with cord expansion and post contrast enhancement	Normal (6)
6	WBC 10/mm^3, protein normal; OCBs not done	Lesions in right cerebellar peduncle, medulla and rest of brainstem; no enhancement	Markedly improved (3)	T2 high signal cervicomedullary junction to T6 and further lesion in conus; cord expansion but no post contrast enhancement	Markedly improved (3)
7	WBC 14/mm^3, protein normal; matched OCBs	Normal	N/D	T2 high signal C4-6 and T2-4 with mild cord expansion and no post contrast enhancement	N/D
8	Normal except OCBs positive	Normal	Normal (20)	Short lesions at C3/4, C5/6, T3 and T12 with mild cord expansion; contrast not given	Poor quality scan (20)
9	Normal; no OCBs	Lesions in pulvinar, subcortical frontal white matter, midbrain and pons	Normal (5)	T2 high signal C1 to conus with cord expansion; contrast not given	Normal (5)

Pt = patient; WBC = white blood cell count; OCBs = oligoclonal bands; CSF = cerebrospinal fluid; MRI = magnetic resonance imaging; CT = computed tomography; FU = follow up; N/D = not done; VEPs = visual evoked potentials; SSEPs = somatosensory evoked potentials.

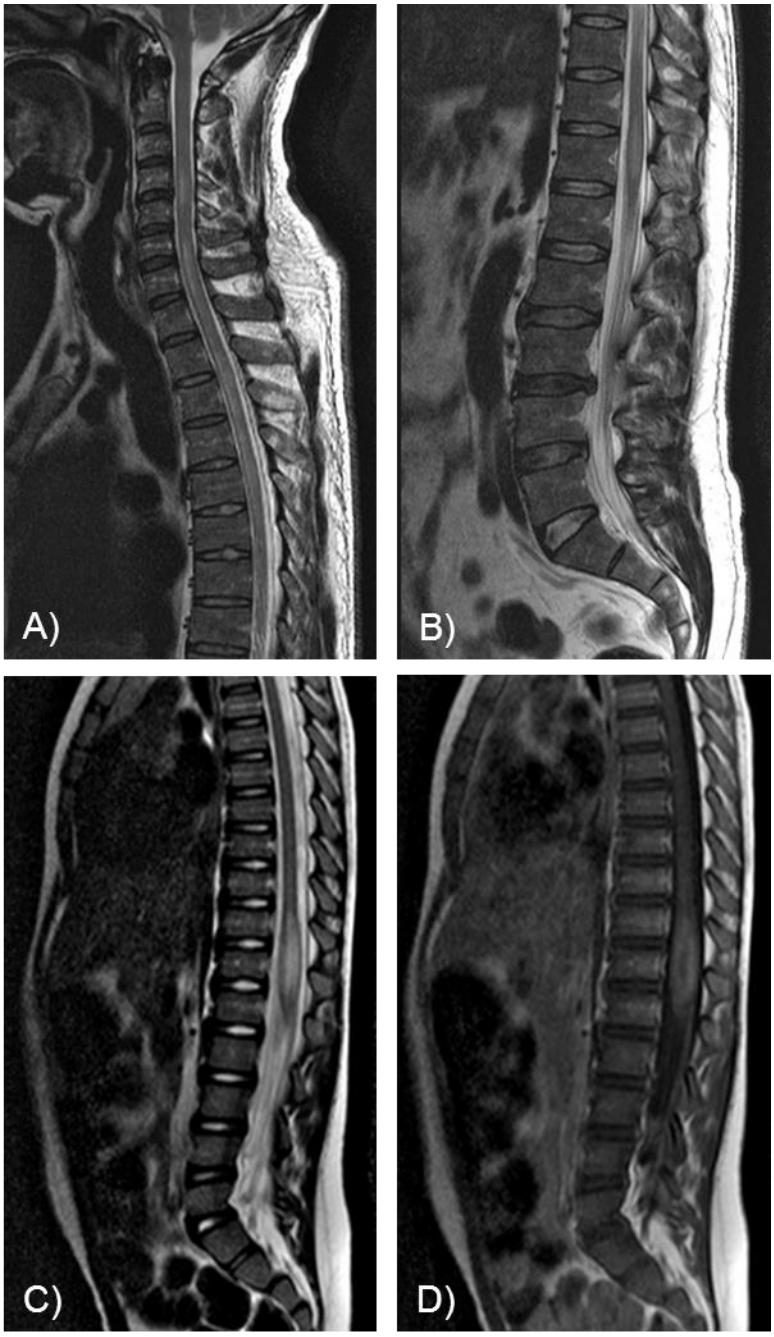


Figure 7.1. Conus involvement in MOG-Ab positive NMO/NMOSD patients. A & B) Sagittal T2 weighted images from patient 6 showing extensive T2 high signal in the cervical and thoracic cord (left) and a further lesion in the conus (right). C & D) T2 weighted (left) and T1 post contrast images (right) from patient 5 showing a lesion in the conus (C) with marked post contrast enhancement (D). Conus involvement in MOG-Ab positive patients was common, being seen in six of eight patients with adequate imaging and occurred significantly more frequently than in patients with AQP4-Ab.

All patients had a brain MRI scan performed during the acute attack. Three patients had completely normal brain MRI scans (with the exception of optic nerve high signal in one), two had non-specific white matter hyperintensities and four patients had ADEM-like brain MRI abnormalities with multiple lesions involving the deep grey matter (Figure 7.2). All six patients with abnormal brain scans at onset had follow-up imaging performed following clinical recovery and five showed complete resolution of all abnormalities (Figure 7.2). The patient with residual signal change had follow-up imaging performed only three months following the acute attack.

CSF examination was performed in all patients, of whom four had a normal examination and five had a lymphocytic pleocytosis. Three of these patients also had an elevated CSF protein concentration. One patient had OCBs in the CSF alone and three had matched OCBs in CSF and serum. No patients had follow-up CSF examination after the acute illness.

Visual evoked potentials (VEPs), when performed, showed delayed latencies in symptomatic eyes only i.e. there was no subclinical optic nerve involvement. VEPs returned to normal when performed at follow-up in three of the four patients with optic nerve involvement and markedly improved in the other patient. Six patients with spinal cord involvement had sensory evoked potentials (SEPs) performed following clinical recovery, and all were normal.

7.3.2 Comparison between MOG-Ab and AQP4-Ab positive NMO/NMOSD patients

7.3.2.1 Comparison of clinical features

Table 7.3 summarises the demographic and clinical features of the two groups. Both groups comprised predominantly Caucasian patients (p=0.431) but there was a significantly higher proportion of males in the MOG-Ab positive group (p=0.016) and there was a trend towards AQP4-Ab positive patients being older at disease onset than MOG-Ab positive patients (mean 44.9 ± 14.8 vs. 32.3 ± 17.1 years; p=0.054).

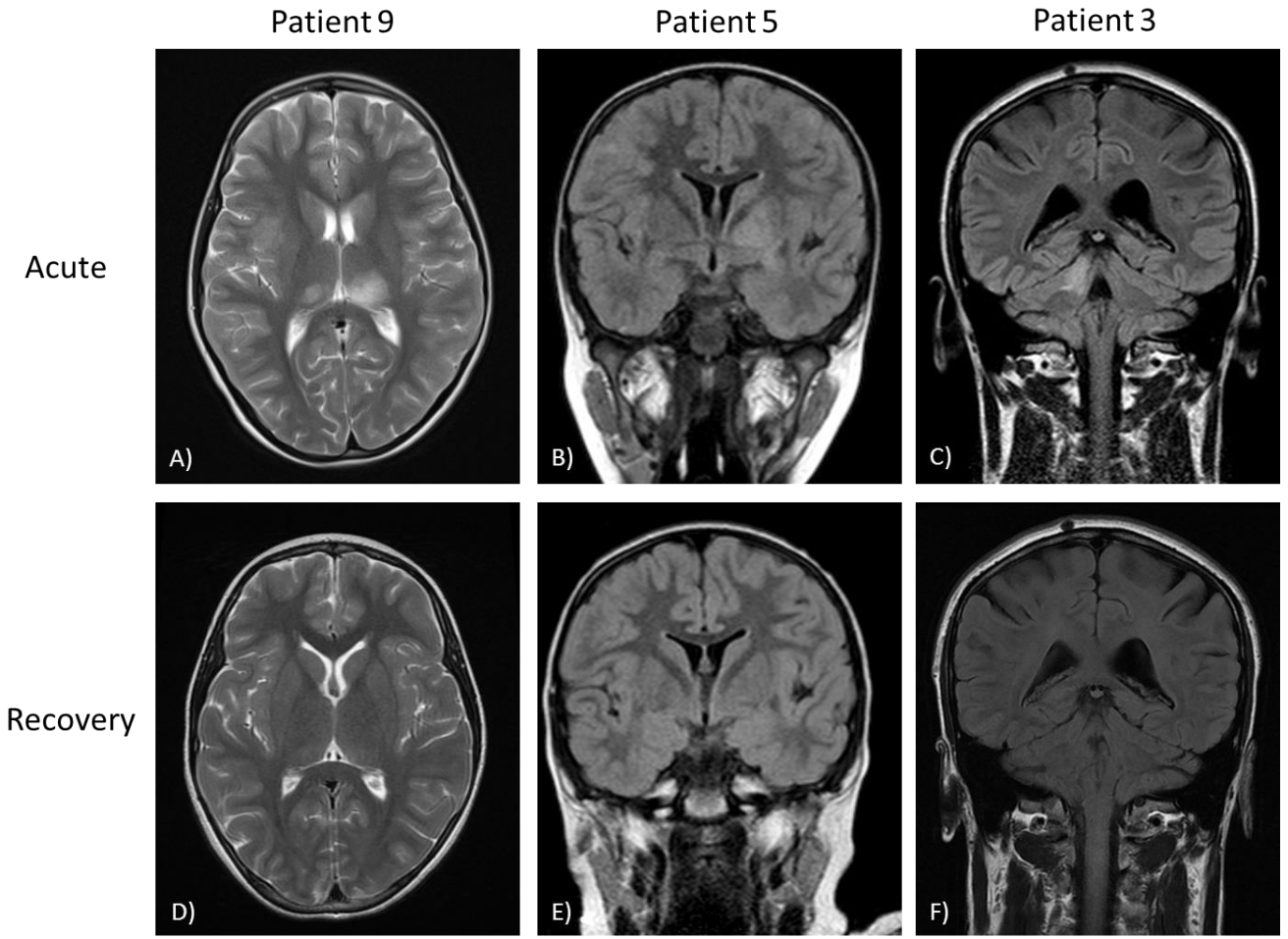


Figure 7.2. MRI brain abnormalities in MOG-Ab positive NMO/NMOSD patients. MRI brain scans in three MOG-Ab positive patients during the acute setting (A-C) and following clinical recovery (D-F). All brain MRI abnormalities completely resolved following clinical recovery, except in patient 6, whose follow-up scan was performed only three months after the acute attack (not shown).

Table 7.3. Comparison of clinical features of MOG and AQP4-Ab positive NMO/NMOSD patients

	MOG-Ab positive (n=9)	**AQP4-Ab positive (n=20)**	**P value**
% females	44	90	0.016
Caucasian ethnicity (%)	78	60	ns
Mean age at onset ± s.d.	32.3 ± 17.1	44.9 ± 14.8	0.054
Onset attack			
ON	0 (0%)	6 (30%)	ns
TM	3 (33%)	12 (60%)	ns
ON + TM	4 (44%)	0 (0%)	0.005
Brain/Brainstem + TM	2 (22%)	0 (0%)	ns
Brain	0 (0%)	2 (10%)	ns
Median nadir EDSS score (range)	6.0 (4 to 8.5)	5.5 (1 to 8.5)	ns
Median EDSS at best recovery (range)	0 (0 to 2.5)	4.0 (0 to 8)	0.010
Number (%) of patients with spinal cord involvement at onset left motor disabled	0 (0%)	7 (58%)	0.007
Number (%) of patients with optic nerve involvement at onset left visually disabled	0 (0%)	2 (33%)	ns
Median follow up, months (range)	18.0 (3.8 to 38.5)	20.5 (5.0 to 36.6)	ns
Subsequent relapse	0 (0%)	8 (40%)	0.033

s.d. = standard deviation; ns = not significant; ON = optic neuritis; TM = transverse myelitis; EDSS = Expanded Disability Status Scale.

Nine AQP4-Ab positive patients had evidence of co-existing autoimmunity (six had co-existing autoimmune disorders with or without autoantibodies and a further three were ANA positive). One MOG-Ab positive patient had a history of hypothyroidism, and none had positive ANA. Two MOG-Ab positive patients had a past history of treated neoplastic disease [breast carcinoma (n=1) and thyroid carcinoma (n=1)].

MOG-Ab positive patients were more likely to present with simultaneous or rapidly sequential ON and TM, whereas AQP4-Ab positive patients usually presented with either ON or TM (p=0.005). Thus, four (44%) MOG-Ab positive patients but no AQP4-Ab positive patients fulfilled the clinical diagnostic criteria for NMO at onset. Onset attacks in both groups tended to be severe and the median EDSS at nadir did not differ between AQP4-Ab positive patients and MOG-Ab positive patients (5.5 vs. 6.0; p=0.435). Four of nine MOG-Ab positive patients and six of 20 AQP4-Ab positive patients had optic nerve involvement at presentation. Where the optic nerves were affected as part of the onset attack, bilateral ON was less common in patients with AQP4-Ab (33% AQP4 vs. 75% MOG) but because of the small numbers this difference was not significant. All affected eyes in both groups had evidence of optic disc swelling (no documentation was available for one patient in each group). ON in both groups tended to be severe. In the MOG-Ab positive group, all patients with optic nerve involvement had visual acuities of less than 6/60 in at least one affected eye and overall, five of seven affected eyes had such severe visual impairment. In the AQP4-Ab positive group, five of six patients had visual acuities of less than 6/60 in at least one affected eye and overall five of eight eyes had acuities of less than 6/60.

Twelve of 20 AQP4-Ab positive patients and all nine MOG-Ab positive patients had spinal cord involvement at disease onset. The median EDSS at nadir in these patients did not differ between AQP4-Ab positive and MOG-Ab positive patients (7.0 vs. 6.0; p=0.803). One notable difference between myelitis attacks in the two groups was that there was a trend towards bladder involvement as the first neurological symptom being more common in MOG-Ab positive patients (33% vs. 0%; p=0.063), whereas sphincter disturbance occurred later in AQP4-Ab positive patients with myelitis.

A high proportion of patients in each group received acute treatment with corticosteroids (100% MOG-Ab positive; 75% AQP4-Ab positive) and a similar proportion in each group additionally underwent plasma exchange (33% vs. 25%) in the acute setting. Despite this, median EDSS at recovery was significantly higher in the AQP4-Ab positive group compared with the MOG-Ab positive group (4 vs. 0; p=0.010) and the median change in EDSS was also significantly greater in the MOG-Ab positive patients (6 vs. 2; p<0.001; Figure 7.3). The five AQP4-Ab positive patients who weren't treated acutely with steroids all made a full (EDSS 0; n=4) or good (EDSS 2; n=1) recovery and thus these untreated patients did not skew the recovery EDSS scores.

Six AQP4-Ab positive patients but no MOG-Ab positive patients were left motor disabled after the onset attack (defined as inability to walk 100 metres unaided) and an additional AQP4-Ab positive patient was left wheelchair dependent. Thus, the proportion of patients with spinal cord involvement left motor disabled after the onset attack was significantly higher in the AQP4-Ab positive group (58% vs. 0%; p=0.007). Two of six (33%) AQP4-Ab positive patients with ON but none of four MOG-Ab positive patients with optic nerve involvement were left visually disabled in one eye (visual acuity <6/36) after the onset attack (p=0.467).

Eighty nine per cent of MOG-Ab positive patients and 65% of AQP4-Ab positive patients were started on immunosuppression at or shortly after disease onset and all AQP4-Ab positive patients were ultimately started on long-term immunosuppression (range 0 to 17 months from onset). Immunosuppression was withdrawn over 9 to 24 months in six of the MOG-Ab positive patients and three remain on low dose tapering steroids whereas all AQP4-Ab positive patients remain on long-term immunosuppression at a therapeutic dose. Despite this, a greater proportion of AQP4-Ab positive patients relapsed over the follow up period (p=0.033) and in fact no MOG-Ab positive patients have subsequently relapsed. The median follow-up duration was similar in the two groups (20.5 AQP4-Ab vs. 18.0 months MOG-Ab; p=0.621).

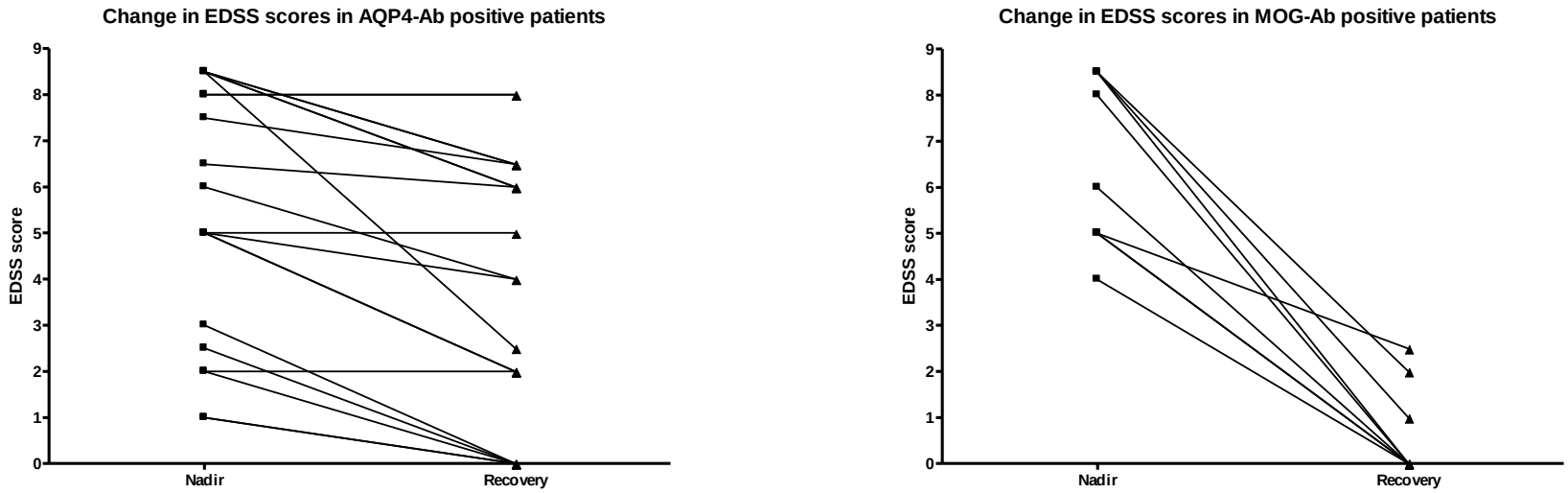


Figure 7.3. EDSS scores at nadir and recovery from the onset attack in NMO/NMOSD patients with AQP4-Ab or MOG-Ab. AQP4-Ab positive patients often showed poor recovery from the acute attack (left), with little change in EDSS scores, whereas recovery in MOG-Ab positive patients was often dramatic (right). Median change in EDSS scores between attack nadir and recovery was significantly higher in MOG-Ab positive patients compared to AQP4-Ab positive patients (6 vs. 2; $p<0.001$).

7.3.2.3 Comparison of radiological and paraclinical features

Twenty one patients (12 AQP4-Ab positive, nine MOG-Ab positive) had clinical evidence of spinal cord involvement at presentation. One AQP4-Ab positive patient with sensory cord symptoms but normal neurological examination had a normal MRI. The spinal cord MRI features of the remaining patients are shown in Table 7.4. The conus was significantly more likely to be involved in patients with MOG-Ab (p=0.012) but there were no differences in terms of frequency of central cord involvement, presence of cord oedema, post contrast enhancement or T1 hypointensity in the two groups and the median length of the longest lesion did not differ between AQP4-Ab positive and MOG-Ab positive patients (p=0.760). Four AQP4-Ab positive patients had follow-up cord imaging available following clinical recovery and there was residual T2 hyperintensity in three patients, compared to two of six MOG-Ab positive patients (p=0.222).

Twenty seven patients (18 AQP4-Ab positive and nine MOG-Ab positive) had an MRI brain scan performed during the acute attack (Table 7.4). MOG-Ab positive patients were significantly more likely to have a scan classified as 'ADEM-like' than patients with AQP4-Ab (44% vs. 0%; p=0.007) and consequently, deep grey matter lesions were more common in MOG-Ab positive patients (p=0.029; Figure 7.4). Surprisingly, lesions adjacent to the fourth ventricle were also more common in MOG-Ab positive patients (p=0.030).

CSF findings were similar in the two groups. There were no differences in the proportions of patients with CSF pleocytosis (50% AQP4-Ab vs. 56% MOG; p=1.000) or elevated protein concentration (25% AQP4-Ab vs. 33% MOG-Ab; p=1.000). Unmatched OCBs were found in only one MOG-Ab positive patient and matched OCBs were found in three of eight MOG-Ab positive patients and four of 12 AQP4-Ab positive patients (p=0.648).

During the acute setting, no MOG-Ab positive patients had delayed VEPs in clinically unaffected eyes. Ten AQP4-Ab positive patients had VEPs performed acutely, of which three of 17 unaffected eyes (in two patients) had asymptomatic delays.

Table 7.4. MRI findings in MOG and AQP4-Ab positive NMO/NMOSD patients

	MOG-Ab positive	AQP4-Ab positive	P value
MRI brain classification	N=9	N=18	
Normal	3 (33%)	7 (39%)	ns
Non-specific	2 (22%)	7 (39%)	ns
MS-like	0 (0%)	2 (11%)	ns
NMO-like	0 (0%)	2 (11%)	ns
ADEM-like	4 (44%)	0 (0%)	0.007
MRI brain lesions	N=9	N=18	
Large-hemispheric	1 (11%)	1 (6%)	ns
Juxta-cortical	2 (22%)	4 (22%)	ns
Deep white matter	1 (11%)	4 (22%)	ns
Adjacent to lateral ventricle	1 (11%)	5 (28%)	ns
Deep grey matter	3 (33%)	0 (0%)	0.029
Pons	3 (33%)	1 (6%)	ns
Medulla	0 (0%)	1 (6%)	ns
Cerebellum	2 (22%)	1 (6%)	ns
Adjacent to 4^{th} ventricle	4 (44%)	1 (6%)	0.030
MRI cord lesions			
Median length of longest lesion, VB (range)	3 (3 to 13)	5 (1 to 17)	ns
Mean length of longest lesion, VB ± s.d.	6.78 ± 5.5	6.25 ± 3.4	ns
T1 hypointensity	5 of 8 (63%)	4 of 10 (40%)	ns
Central grey matter involvement	5 of 6 (83%)	9 of 11 (82%)	ns
Post contrast enhancement	2 of 6 (33%)	7 of 8 (88%)	ns
Cord oedema	9 (100%)	11 (100%)	ns
Sagittal location			
Involving cervical cord	6 of 9 (66%)	9 of 11 (82%)	ns
Involving thoracic cord	8 of 9 (89%)	8 of 11 (73%)	ns
Involving conus	6 of 8 (75%)	2 of 11 (18%)	0.024

N = number; s.d. = standard deviation; ns = not significant; MRI = magnetic resonance imaging; MS = multiple sclerosis; ADEM = acute disseminated encephalomyelitis; NMO = neuromyelitis optica; MOG-Ab = myelin oligodendrocyte glycoprotein antibody; AQP4-Ab = aquaporin-4 antibody; VB = vertebral bodies.

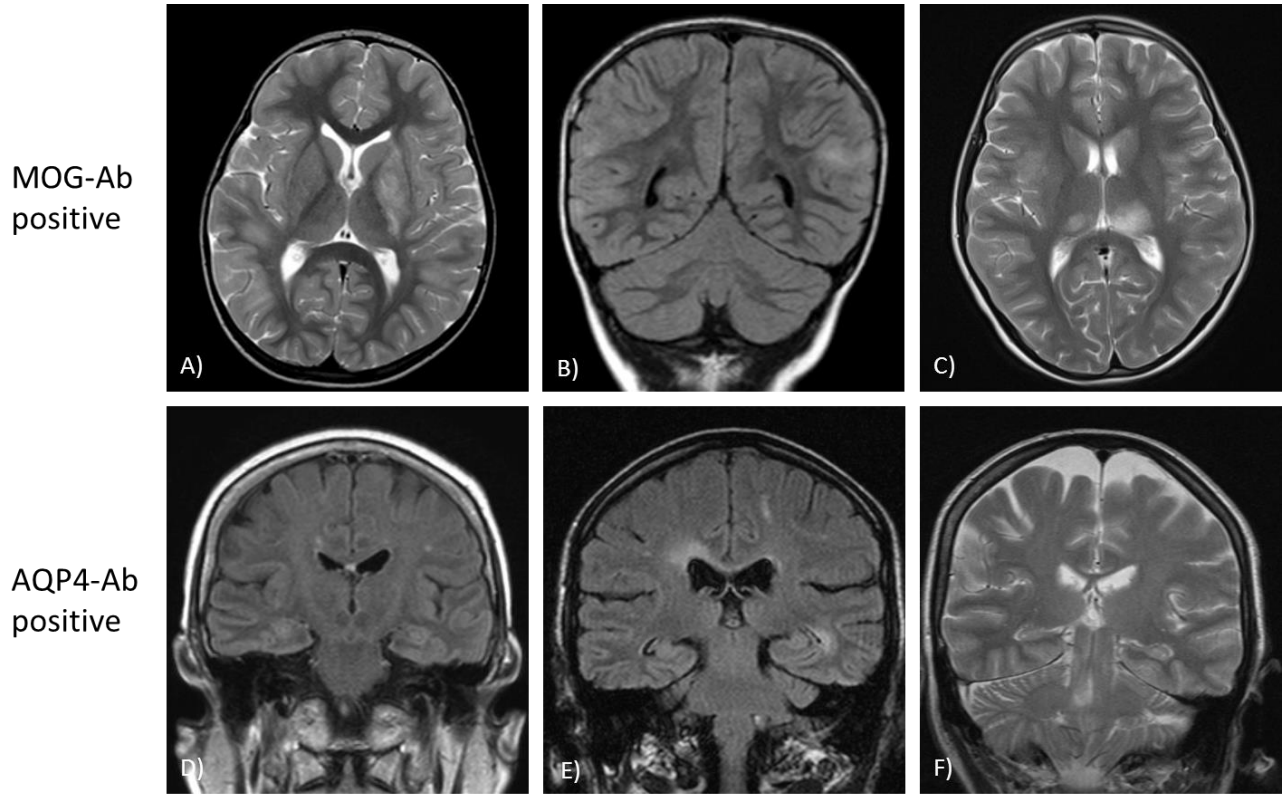


Figure 7.4. MRI brain abnormalities in MOG-Ab positive and AQP4-Ab positive patients. Brain abnormalities in MOG-Ab positive patients were often ADEM-like (A-C). A) T2 hyperintensity in the left basal ganglia in patient 5. The same patient also had multiple fluffy T2 juxta-cortical hyperintensities (B), some of which demonstrated post-contrast enhancement. C) Bi-thalamic T2 high signal abnormalities in patient 9. Brain abnormalities in AQP4-Ab positive patients tended to be more non-specific (D-F) with small well-demarcated subcortical and deep white matter lesions (D) and peri-ventricular lesions (E). F) Pontine lesion at the floor of the 4th ventricle, considered characteristic of AQP4-Ab disease. However, lesions adjacent to the 4th ventricle were actually seen more frequently in patients with MOG-Ab.

All patients had repeat AQP4-Ab and MOG-Ab testing following clinical recovery. Five MOG-Ab positive and three AQP4-Ab positive patients (all low positive in the acute setting) have seroconverted, with no detectable antibodies during disease remission.

7.4 Discussion

We have found MOG-Ab in some patients previously considered to have AQP4-Ab seronegative NMO/NMOSD (Kitley, Woodhall et al, 2012), and in this chapter the clinical and paraclinical features of these patients have been characterised in detail. These patients share common clinical features, but demonstrate some differences when compared to NMO/NMOSD patients with AQP4-Ab.

MOG is a CNS-specific antigen that is expressed on the outer surface of myelin sheaths. As mentioned, its location therefore makes it readily accessible to autoantibodies in the extracellular space, and antibodies to MOG are potentially pathogenic. In animal models, MOG monoclonal antibodies augment the severity and duration of EAE (Linington, Bradl et al, 1988) and immunisation with MOG peptides leads to monophasic, relapsing-remitting or chronic EAE with histological evidence of inflammation and demyelination (Adelmann, Wood et al, 1995; Johns, Kerlero de Rosbo et al, 1995; Devaux, Enderlin et al, 1997; Storch, Stefferl et al, 1998; Sakuma, Kohyama et al, 2004; Collongues, Chanson et al, 2012). An NMO-like disease with selective distribution of inflammatory lesions in the optic nerves and spinal cord can be induced in double-transgenic mice in whom MOG-specific T and B cells are present through crossing MOG-specific T cell receptor transgenic mice with MOG-specific Ig-heavy chain knock-in mice (Bettelli, Baeten et al, 2006; Krishnamoorthy, Lassmann et al, 2006).

MOG-Ab in humans were first investigated in MS and clinically isolated syndrome (CIS). MOG-Ab seropositivity was reported in 20 to 50% patients with relapsing-remitting MS (Kuhle, Lindberg et al, 2007) or CIS (Rauer, Euler et al, 2006; Pelayo, Tintore et al, 2007; Pittock, Reindl et al, 2007) and there were suggestions that MOG-Ab seropositivity in CIS patients increased the risk of conversion to clinically-definite MS (Berger, Rubner et al, 2003), though this was not confirmed in a later publication by the same group (Pittock, Reindl et al, 2007). However,

because these methods used denatured or linear MOG epitopes, they detected antibodies that bound to different MOG epitopes from those detected by CBAs using native MOG (Lalive, Menge et al, 2006b). Antibodies to native MOG are believed to be pathogenic whereas those that detect denatured or synthetic fragments are not (Brehm, Piddlesden et al, 1999; Von Büdingen, Hauser et al, 2004). The methods used in these studies were therefore not reliable for detecting pathogenic MOG-Ab (Pittock, Reindl et al, 2007), thus questioning the significance of their results.

Using techniques to detect antibodies to conformationally-expressed MOG i.e. pathologically relevant antibodies, subsequent studies [with one exception (Zhou, Srivastava et al, 2006)] have found that MOG-Ab are rare in adults with MS or CIS and when present occur only at low titre (O'Connor, McLaughlin et al, 2007; Brilot, Dale et al, 2009; McLaughlin, Chitnis et al, 2009; Di Pauli, Mader et al, 2011). However, MOG-Ab are seen more frequently in childhood demyelinating disorders (O'Connor, McLaughlin et al, 2007; Brilot, Dale et al; 2009; Selter, Brilot et al, 2010; Di Pauli, Mader et al, 2011; Lalive, Hausler, et al 2011), particularly ADEM, in which high titre MOG-Ab have been found in up to 47% of patients (O'Connor, McLaughlin et al, 2007; Brilot, Dale et al; 2009; Selter, Brilot et al, 2010; Di Pauli, Mader et al, 2011; Lalive, Hausler, et al 2011). The phenotype of MOG-Ab positive childhood ADEM has not been described in sufficient detail to establish if it is 'Devic's/NMO-like' or indistinguishable from classical ADEM. The findings of these studies are summarised in Table 7.5. Some studies have shown that in paediatric ADEM, MOG-Ab titres decrease or disappear over time following clinical recovery, consistent with a pathogenic role (Di Pauli, Mader et al, 2011; Probstel, Dornmair et al, 2011), whereas titres in MS patients tend to be low and remain stable or fluctuate over time (Di Pauli, Mader et al, 2011; Probstel, Dornmair et al, 2011).

As mentioned in previous chapters, the original description of Devic's disease, that of monophasic simultaneous or rapidly sequential TM and ON (Devic, 1894) is relatively rare in AQP4-Ab positive NMO, but higher rates have been reported when AQP4-Ab seronegative patients are included in cohorts (Rivera, Kurtzke et al, 2008; Bichuetti, Oliveira et al, 2009; Collongues, Marignier et al, 2010). Such monophasic multifocal inflammation within the CNS is more reminiscent of ADEM. In our cohort, four of nine MOG-Ab positive patients had a 'classic'

Table 7.5. Summary of publications reporting MOG-Ab in CNS demyelinating disorders

Study	Patients	Assay	% MOG-Ab seropositivity
Zhou, Srivastava et al, 2006	163 MS patients (age not specified)	FACS	32% of MS patients but scores were generally low
O'Connor, McLaughlin et al, 2007	Adult and paediatric ADEM, MS and CIS	RIPA	13 of 69 (19%) ADEM patients 1 of 109 (1%) adult MS and 0 of 32 (0%) adult CIS patients 1 of 19 (5%) paediatric MS patients 0 of 58 (0%) viral encephalitis patients and 1 of 75 (1%) healthy controls
Brilot, Dale et al, 2009	47 paediatric patients (ADEM n=19; CIS n=28) 54 adult MS controls	FACS and CBA	47% patients (ADEM and CIS) and 7% controls. High titre MOG-Ab were seen in 40% of patients but in no controls
McLaughlin, Chitnis et al, 2009	131 paediatric MS 254 adult MS	FACS	21% paediatric MS 4% adult MS patients; seen only at low levels
Selter, Brilot et al, 2010	44 paediatric patients with a 1st demyelinating event	FACS	9 of 19 (47%) ADEM patients 9 of 25 (36%) CIS patients, some of whom progressed to MS
Lalive, Hausler et al, 2011	11 paediatric ADEM, 22 paediatric MS, 7 encephalitis controls	ELISA and FACS	Overall, 12% of the demyelinating group were MOG-Ab positive; 27% ADEM, 5% MS and 0% controls
Di Pauli, Mader et al, 2011	34 ADEM (27 paediatric, 7 adult) 38 CIS (18 paediatric, 20 adult) 89 MS (18 paediatric, 71 adult)	CBA	44% ADEM, 8% CIS, 2% MS, 2% controls. All CIS patients with high titre MOG-Ab had ON or TM
Probstel, Dornmair et al, 2011	54 paediatric ADEM, 65 paediatric MS, 7 paediatric CIS, 62 adult MS	FACS	35% paediatric ADEM, 29% paediatric CIS, 15% paediatric MS, 6% adult MS

MS = multiple sclerosis; ADEM = acute disseminated encephalomyelitis; CIS = clinically isolated syndrome; FACS = fluorescence-activated cell sorting; FIPA = fluorescence immunoprecipitation assay; CBA = cell-based assay; ELISA = enzyme-linked immunosorbent assay; MOG-Ab = myelin oligodendrocyte glycoprotein antibodies; EAE = experimental autoimmune encephalomyelitis; CSF = cerebrospinal fluid.

Devic's phenotype with simultaneous or sequential ON and LETM, and a further two had LETM with brain lesions and mild encephalopathy. Nearly half of the MOG-Ab positive patients fulfilled clinical NMO diagnostic criteria at presentation. These findings suggest that MOG-Ab NMO/NMOSD may account for some cases of Devic's disease as it was originally described and that MOG-Ab positive NMO/NMOSD may overlap with ADEM.

Although eight of the nine patients with MOG-Ab had NMO/NMOSD and shared some common features with AQP4-Ab positive NMO/NMOSD patients, there were some notable differences. AQP4-Ab disease has a striking female preponderance. By contrast, the majority of MOG-Ab patients were male. There was also a trend towards the MOG-Ab positive patients being younger at disease onset. Although MOG-Ab positive patients had severe onset attacks, with comparable nadir EDSS scores to AQP4-Ab positive patients, they showed remarkable recovery, and EDSS scores at follow-up were significantly lower than those with AQP4-Ab and no MOG-Ab patients were left with significant visual or motor disability. Moreover, MRI abnormalities resolved completely on follow-up imaging in most patients and evoked potentials normalised in the majority. In our experience, evoked potentials tend to remain abnormal and spinal cord imaging usually shows persistent abnormal signal change and atrophy in patients with AQP4-Ab disease, though few AQP4-Ab patients in this cohort had follow-up studies. Moreover, no MOG-Ab positive patients have relapsed following the onset attack, although immunosuppressive therapy has not yet been totally withdrawn in three. Nevertheless, despite the relatively short follow-up period, a substantial proportion of AQP4-Ab positive patients relapsed despite similar use of immunosuppression. Thus, taken together, these findings suggest that MOG-Ab positive patients have more favourable outcomes than patients with AQP4-Ab, and may not require long-term immunosuppression.

Interestingly, there are some clues in experimental models as to why patients with MOG-Ab may have less severe disease than patients with AQP4-Ab. Firstly, although immunisation of rodents with MOG peptides leads to inflammation and demyelination (Adelmann, Wood et al, 1995; Johns, Kerlero de Rosbo et al, 1995; Devaux, Enderlin et al, 1997; Storch, Stefferl et al, 1998; Sakuma, Kohyama et al, 2004; Collongues, Chanson et al, 2012), there is relative preservation of axons

(Storch, Stefferl et al, 1998; Sakuma, Kohyama et al, 2004) and astrocytes (Bradl, Misu et al, 2009). Secondly, in fetal rat brain cultures, incubation with a monoclonal MOG-Ab and complement results in demyelination, but a much lesser degree of oligodendrocyte loss (Kerlero de Rosbo, Honegger et al, 1990), suggesting that demyelination induced by MOG-Ab does not necessarily lead to oligodendrocyte death, and changes may be reversible. Finally, as was noted in our patients, MRI scans in MOG-EAE animal models show T2 hyperintensity in the optic nerves, spinal cord and periventricular regions, which resolve completely following clinical recovery (Collongues, Chanson et al, 2012).

The pathogenic role of human MOG-Ab has not yet been definitively proven, in that they have not yet been shown to produce demyelination in passive transfer experiments. However, as mentioned in chapter 6, there is accumulating evidence that MOG-Ab have pathogenic potential. Animal models have already shown that T and B cell responses to MOG can induce an NMO-like illness and there is accruing experimental evidence that MOG-Ab are pathogenic. Sera from MOG-Ab positive patients can cause death of MOG-transfected cells *in vitro* (Zhou, Srivastava et al, 2006; Brilot, Dale et al, 2009) and antibodies in these sera bind to myelin in rat brain sections (Zhou, Srivastava et al, 2006) and to normal human white matter (Brilot, Dale et al, 2009). MOG-Ab have been shown to be predominantly of the IgG1 subtype (McLaughlin, Chitnis et al, 2009; Mader, Gredler et al, 2011), a subtype that can fix complement and MOG-Ab positive patient sera have been shown to cause complement deposition on membranes of MOG-transfected cells (Mader, Gredler et al, 2011).

Since the work in this thesis was performed, there have been several publications by a single group (Mader, Gredler et al, 2011; Rostasy, Mader et al 2012; Huppke, Rostasy et al, 2013; Rostasy, Mader et al, 2013) also reporting the presence of MOG-Ab in NMO/NMOSD (Table 7.6). The first of these (Mader, Gredler et al, 2011), which used a CBA, screened patients with CNS demyelinating diseases and found MOG-Ab to be common in ADEM (42% tested) but also to be present in NMO (7%), NMOSD (13%), CIS (6%), MS (3%) and neurological controls (3%). All groups included both adults and children. Titres in NMO/NMOSD and ADEM were higher than in MS and CIS but median MOG-Ab titres did not differ between NMO, NMOSD and ADEM.

Table 7.6. Summary of clinical studies describing MOG-Ab in NMO/NMOSD

Study	N	Ethnicity	Age (years)	Gender F:M	Phenotype	MRI	Outcome	Pathogenicity/other comments
Mader, Gredler et al, 2011	10	N/A	Adult and paediatric (2 to 56)	7:3	rNMO (n=3); rON (n=5); mLETM (n=2)	None had brain lesions	8 of 10 followed relapsing course	IgG1 subtype and complement activating

Rostasy, Mader et al, 2012	17	N/A	All paediatric	N/A	mON (n=2), rON (n=10);
					rON+ADEM (n=2);
					ON in MS (n=3)
						ADEM-like abnormalities; resolved at follow-up
						LETM in 2 patients with rON
							N/A	Titres in MS were low

Huppke, Rostasy et al, 2013	7	6/7 Caucasian	Paediatric (4 to 8)	5:2	ADEM then mON or rON	White and grey matter involvement, including basal ganglia; good resolution at follow-up	Mild residual visual disability in 4 of 7. Good response to steroids	Titres remained high over time
Rostasy, Mader et al, 2013	3	N/A	Paediatric (3 to 14)	3:0	rNMO	Patient 1: lesion in cerebellum; LETM; resolved at follow-up Patient 2: lesion in basal ganglia; cervical LETM Patient 3: multiple brain lesions including basal ganglia; LETM involving conus	Good outcome with steroids	No co-existing autoantibodies

N = number; N/A = not available; F = female; M = male; NMO = neuromyelitis optica; ON = optic neuritis; LETM = longitudinally extensive transverse myelitis; m = monophasic; r = relapsing; ADEM = acute disseminated encephalomyelitis; MS = multiple sclerosis; MRI = magnetic resonance imaging.

The NMO/NMOSD patients positive for MOG-Ab were a heterogeneous group comprising eight adults and four children and included patients with relapsing NMO (n=3), recurrent ON (n=5) and monophasic LETM (n=2). One patient was double positive for both AQP4-Ab and MOG-Ab. No information was provided on disease duration, whether samples were taken during relapse or remission, or any treatment data. The MOG-Ab CBA used a high cut-off of 1:160 to define seropositivity, which may suggest that they had problems with non-specific binding.

The three later studies by this same group have focussed on paediatric patients. One publication described a paediatric phenotype of ADEM followed by ON (Huppke, Rostasy et al, 2013). All patients responded well to steroids but four have been left with mild visual impairment; none have marked disability. Interestingly, as was noted in our patients, MRI changes resolved or markedly improved at follow-up in all patients. In another publication, they screened a cohort of paediatric patients with a history of ON (Rostasy, Mader et al 2012), finding MOG-Ab in patients with monophasic and relapsing ON (some of whom had ADEM-like brain scans and/or LETM on spinal imaging) and patients with ON in the context of MS or CIS with an MS-like MRI. Titres in the first two groups were significantly higher than in MS/CIS patients. Finally, this Austrian group screened 106 paediatric patients with a CNS demyelinating syndrome for MOG-Ab (Rostasy, Mader et al, 2013). Of eight patients fulfilling the 2006 Wingerchuk NMO diagnostic criteria, two were positive for AQP4-Ab and three were positive for MOG-Ab. MOG-Ab were also seen in patients with monophasic and recurrent ON (14% and 67%), ADEM (45%), CIS (3%) and MS (9%). The only monophasic NMO patient had neither MOG-Ab nor AQP4-Ab. The three NMO patients who tested positive for MOG-Ab all had high titre antibodies, which persisted at follow-up, albeit at lower levels during remission. All patients made an excellent and prompt recovery with steroids.

Although these studies found MOG-Abs in some patients with NMOSD, in contrast to our findings, some patients had relapsing disease and low titres of MOG-Abs were also found in a small number of adult patients with MS or other neurological conditions. This may reflect differences in the assays used but it does appear that MOG-Abs, particularly at low titre, may lack

specificity. Within our cohort, patient 8 stood out as being different from the other patients, who all shared common clinical features. Patient 8 was the only patient with short cord lesions and intrathecal OCB synthesis, and clinically resembled MS more than NMO or ADEM. She had low level MOG-Ab which disappeared over time. Although some of the other patients also had low-level MOG-Ab, they shared similar characteristics to the patients with higher MOG-Ab levels. Thus, it may be that at high titre, MOG-Ab are specific for an NMO/NMOSD phenotype with some overlap with ADEM, whilst low titre antibodies, although sometimes associated with this same phenotype, are less specific and may occur as a secondary phenomenon in other diseases. We are aware of some children testing positive for MOG-Ab on the Oxford CBA with other phenotypes such as recurrent ataxia or ADEM followed by recurrent ON, and are also aware of a single MOG-Ab positive adult patient with relapsing NMO followed in another UK centre. Thus, although the MOG-Ab positive patients described here all had a monophasic illness, it is possible that some may relapse over a longer follow-up period. Nevertheless, these patients seem to have more favourable outcomes than NMO/NMOSD patients with AQP4-Ab.

In summary, we have found MOG-Ab in a group of AQP4-Ab seronegative NMO/NMOSD patients who largely share similar disease characteristics. In particular, the clinical and radiological recovery from what was typically a severe acute illness were remarkable and risk of relapsing disease appears much lower than in AQP4-Ab disease. Testing for MOG-Ab in patients with NMO/NMOSD without AQP4-Ab is important, since their presence may suggest a more benign illness, with a requirement for acute treatment with prednisolone but without the need for long-term immunosuppression. However, further studies are required to fully characterise the role of MOG-Ab disease and determine their place in the spectrum of NMO.

Chapter 8: Conclusions and future directions

8.1 Conclusions

Historically, NMO has been defined clinically as the co-association of ON and LETM and the cohorts of most clinical NMO studies have used diagnostic criteria that rely heavily on this co-association. More recently, the trend has shifted towards defining NMO by AQP4-Ab seropositivity since observations suggest that this disease biomarker generally confers a severe, relapsing and potentially disabling disease course, which may differ from that of seronegative patients. The work in this thesis, together with developments in AQP4-Ab assays in the Oxford laboratory, have contributed towards this trend. In this thesis the spectrum of AQP4-Ab disease has been characterised, and clinical and experimental investigations of patients with clinical features of NMO/NMOSD but no AQP4-Ab have revealed that many of these patients have alternative diagnoses, demonstrate differences when compared to AQP4-Ab patients, and should be considered separately from those with AQP4-Ab disease.

In characterising the clinical features and disease course of a large cohort of consecutively identified AQP4-Ab positive patients, we have demonstrated that AQP4-Ab disease is not synonymous with the term NMO, since at disease presentation only 4% fulfilled Wingerchuk NMO diagnostic criteria and even at last follow-up nearly half of patients had limited or atypical disease phenotypes and did not fulfil these criteria. This is in part likely related to treatment practices, but nevertheless it appears that disease in some patients has a particular predilection for one anatomical site, with some patients experiencing exclusive involvement of either the spinal cord or optic nerves. The clinical outcomes and prognostic characteristics of patients with AQP4-Ab disease have not previously been well characterised; previous studies have included only patients with the full NMO phenotype and many also included AQP4-Ab seronegative patients.

We have shown that the majority of patients with AQP4-Ab follow a relapsing course, with a high risk of relapse within the first two years of disease onset. Moreover, relapse-related morbidity and mortality are substantial. Ethnicity and age at disease onset appear to be important predictors of

visual and motor disability and mortality. Young-onset patients in the UK, but not in the Japanese cohort, commonly presenting with ON, had a high risk of visual disability whilst older patients in both cohorts had a high risk of motor disability, regardless of their onset symptom. Genetic factors also appeared important. The UK cohort seemed to have more severe disease than the Japanese cohort, with more severe onset attacks, a higher relapse frequency and greater disability at follow-up, despite earlier immunosuppression. Moreover, within the UK cohort, there were differences between ethnic groups, with Afro-Caribbean patients having a younger age at disease onset, more brain and multifocal attacks and higher likelihood of visual disability than Caucasian patients. Thus, this work suggests that age at disease onset and genetic factors are both likely to be important in determining clinical outcomes in AQP4-Ab disease. These features are useful for counselling and management of patients and also have implications for interpreting clinical studies; outcomes appear not to be generic across populations and may need to be tailored to individual cohorts.

Having demonstrated clinical heterogeneity amongst AQP4-Ab positive patients, we explored whether immunological factors, driven by ethnicity, age or other patient factors may be involved in the differences observed in disease course and phenotype. Specifically, we investigated AQP4-Ab binding specificity to the two AQP4 isoforms in a group of clinically well-characterised patients. However, although relative binding to the two AQP4 isoforms differed between patients, we could not identify a relationship between these differences and the clinical features of disease such as severity, anatomical site of attack, onset age or ethnicity. Additionally, we could not identify a threshold AQP4-Ab level for relapses, either across the group or within individual patients. Definite relapses could occur without a rise in antibody levels, and high antibody levels did not necessarily result in disease activity. Moreover, in one patient for whom stored sera were available, AQP4-Ab were detectable many years prior to clinical onset of disease, as has been reported in other patients (Leite, Coutinho et al, 2012). These findings suggest that the pathogenesis of NMO and the occurrence of relapses must be related to factors other than just AQP4-Ab. It may be that T cells and other inflammatory mediators are crucial to the pathogenesis,

or simply that AQP4-Ab can only exert their effects when other factors enable their passage into the CNS through periodic breakdown of the blood-brain barrier.

Despite advances in the development of AQP4-Ab assays, a variable proportion of patients with clinical characteristics of NMO/NMOSD are AQP4-Ab seronegative. It is unclear whether these patients represent a distinct clinical subgroup of NMO or have a condition other than NMO such as MS or ADEM. Previous studies looking at the features of seronegative NMO/NMOSD patients have yielded conflicting results, in part because most used older less sensitive assays to define cohorts and thus likely included false negative cases. Using the sensitive Oxford CBA to define AQP4-Ab status, we found 'true' seronegative NMO and NMOSD to be rare. AQP4-Ab seronegative patients represented an aetiologically heterogenous group and the majority had alternative identifiable diagnoses. Thus, unsurprisingly, these patients demonstrated differences when compared to those with AQP4-Ab disease. Nevertheless, these differences, which included younger age at onset, higher proportion of males, lower frequency of co-existing autoimmune disorders and greater probability of experiencing simultaneous/rapidly sequential ON/TM at disease onset, could be useful in differentiating AQP4-Ab disease from other disorders in the acute setting before results of investigations become available.

We found MOG-Ab in a significant proportion of AQP4-Ab seronegative NMO/NMOSD patients. In patients seen consecutively within our clinical NMO service over a three year period, AQP4-Ab positivity was only twice as common as MOG-Ab positivity. Although a pathogenic role for MOG-Ab has not yet been proven, these patients shared similar disease characteristics and demonstrated differences when compared to AQP4-Ab positive patients. The MOG-Ab patients all had myelitis at onset with or without ON and/or encephalopathy and clinically most would have been classed as ADEM or monophasic NMO. Despite often experiencing severe onset attacks, these patients demonstrated remarkable clinical, radiological and neurophysiological recovery and the risk of relapsing disease appeared lower than in those with AQP4-Ab. MOG-Ab positive patients were also younger at disease onset and included a high proportion of males. These are features that have been reported in other AQP4-Ab seronegative NMO cohorts and it is possible

that some of these patients may have MOG-Ab. MOG-Ab seropositivity appears to confer more favourable outcomes than AQP4-Ab positivity but clearly the features of MOG-Ab disease now need studying in larger cohorts with longer follow-up durations to define the role of MOG-Ab in the spectrum of NMO. It is important to realise that some MOG-Ab assays appear to report lower specificity to the phenotype we report here and thus the interpretation of the results at present appears to be laboratory specific.

Experimental studies using AQP4-Ab seronegative NMO/NMOSD patient sera also suggested that seronegative patients represent an aetiologically heterogeneous group. Some had low levels of AQP4-Ab, whilst others had MOG-Ab and one patient had GlyR-Ab; antibodies to GlyR have also been found in samples sent for AQP4-Ab testing through the Oxford diagnostic assay service, and in seronegative patients in Turkey. Thus, GlyR-Ab may also be relevant in seronegative NMO and this warrants further investigation. Samples from a small number of seronegative patients bound to neurons in primary culture and samples from one patient bound to oligodendrocytes. Thus, these patients may have antibodies to as yet undetermined antigens and NMO may comprise patients with autoantibodies against a number of different targets, as has been seen in other autoimmune neurological disorders such as MG and autoimmune encephalitis.

Overall, the work in this thesis has demonstrated that AQP4-Ab seronegative NMO/NMOSD patients represent a clinically and aetiologically heterogeneous group and that these patients should therefore be classed separately from those with AQP4-Ab.

8.2 Further work

We have shown that a significant proportion of AQP4-Ab seronegative NMO/NMOSD patients have MOG-Ab. The pathogenicity of MOG-Ab has not yet been proven and although MOG can cause demyelinating disease in rodents, it will be important to investigate whether sera from NMO patients with MOG-Ab can cause disease in passive transfer experiments. Our clinical observations suggest that MOG-Ab positive patients have more favourable outcomes than AQP4-Ab positive patients and it will be interesting to investigate potential pathogenic mechanisms that

may underlie this. Experimental work has suggested that there is relative preservation of axons and astrocytes in EAE induced with MOG peptides and if this is also seen in passive transfer experiments using human MOG-Ab, this may explain why inflammation in MOG-Ab disease is reversible.

The Oxford MOG-Ab CBA is now being used as a UK diagnostic assay and positive results have now been found in over 90 patients. The clinical features of these patients now need to be obtained to establish whether the phenotype associated with MOG-Ab is as characteristic as our preliminary observations suggest.

The seronegative experimental work performed in this thesis suggests that some AQP4-Ab negative NMO/NMOSD patients may have low titre AQP4-Ab, since FACS identified three patients who were low positive, one of whom has subsequently also tested positive on the routine diagnostic CBA. Agrin, a heparan sulfate proteoglycan that is a component of the extracellular matrix, is involved in clustering acetylcholine receptors at the motor end plate of the neuromuscular junction and there is some evidence that agrin is also involved in clustering of AQP4 (Noell, Fallier-Becker et al, 2007; Noell, Fallier-Becker et al, 2009; Fallier-Becker, Sperveslage et al, 2011). It would be interesting to establish whether these findings are true, and if so, whether clustering of AQP4 by agrin can improve the sensitivity of the AQP4 CBA to potentially identify very low levels of AQP4 in the sera of AQP4-Ab negative NMO/NMOSD patients.

The seronegative experimental work also identified some patients whose sera bound to neurons and/or oligodendrocytes. Immunoprecipitation experiments were unable to identify the target antigen and further work is clearly needed to try and identify novel antigen targets in seronegative NMO.

Some sera from seronegative NMO/NMOSD patients bound weakly to astrocytes. Further work to ascertain the significance of this might include experiments to determine whether these samples could activate complement on the cell surface of astrocytes and cause astrocyte damage and death.

Additionally, sera from such patients could be used in animal models to establish whether the pattern of abnormalities seen with intracerebral injection of sera mimics that seen with AQP4-Ab positive sera.

Finally, the anatomical restriction of clinically significant disease in NMO remains a mystery, given that AQP4 is expressed throughout the CNS and other tissues. This may be related to many factors such as the differential expression of the two AQP4 isoforms, antigen accessibility or the relative frequency of complement inhibitor proteins and these factors need exploring.

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