Epidemiological, clinical and immunological aspects of neuromyelitis optica (NMO)

NMO

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• Paper I: Asgari N, Lillevang ST, Skejoe HP, M. Falah, MD Stenager E, Kyvik KO. A population-based study of neuromyelitis optica in Caucasians. Neurology 2011; 76: 1589-1595.

• Paper II: Asgari N, Nielsen C, Stenager E, Kyvik KO, Lillevang ST. HLA, PTPN22 and PD-1 associations as markers of autoimmunity in neuromyelitis optica. Mult Scler 2012; 18:23-30.

• Paper III: Asgari N, Khorooshi R, Lillevang ST, Owens T. Complement-dependent pathogenicity of brain-specific antibodies in cerebrospinal fluid. J Neuroimmunol. 2013 Jan 15;254(1-2):76-82.

INTRODUCTION

Optic neuritis (ON) and transverse myelitis (TM) were recognized and described as disease entities in 1870 by Albutt (cited by (2)) and shortly after by Erb (3). The classical definition of NMO originates from Gault and Devic who in 1894, on the basis of 17 cases, characterized NMO as an acute, fulminant, monophasic disorder consisting of ON and TM occurring simultaneously or in rapid succession (4) . This definition is also called Devic's classical syndrome. Later a relapsing form of NMO was reported, recognizing the existence of two NMO subtypes (5).
NMO has been the subject of intense scientific and clinical interest during the last few years, leading to the recognition of a more heterogeneous clinical presentation, mainly due to the discovery of serum immunoglobulin G autoantibodies towards the water channel aquaporin-4 (AQP4) in the majority of NMO patients (6). NMO is an inflammatory demyelinating disease (IDD) (Fig. 1) of the central nervous system (CNS) and probably the most common non-MS IDD in the CNS (7). Clinical, neuroimaging, immunological and histopathological characteristics have been identified, which led to recognition of NMO as a distinct entity different from MS and other IDDs (8-10). Lennon et al. (2005) demonstrated that IgG from an AQP-4 antibody–positive NMO patient, called NMO-IgG, binds selectively to AQP4 in astrocytic foot processes at the abluminal face of microvessels, pia, subpia, and Virchow-Robin sheath of normal mouse CNS. Multiple aspects may thus contribute to understanding of NMO including clinical, epidemiological, genetic and immunological factors.
In this PhD thesis the terms NMO-IgG or anti-AQP4 antibodies are used when the assay principle is specifically mentioned in the reference, whereas anti-AQP4 antibodies/NMO-IgG is used as a general term.

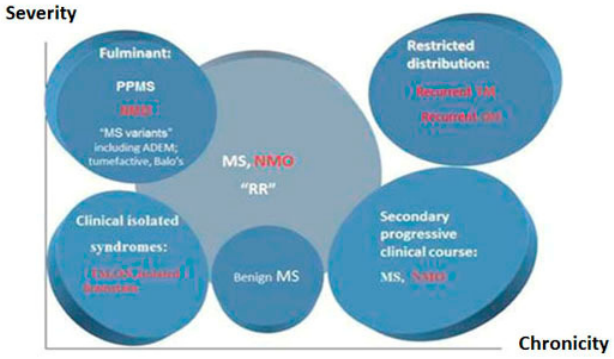

Figure 1
Fig.1. Inflammatory demyelinating diseases of the CNS (IDD). Adapted from Weinshenker 2011, with permission.
MS = multiple sclerosis, NMO= Neuromyelitis optica, PPMS= Primary Progressive MS, ON = optic neuritis, RR= relapsing-remitting curse, TM = acute transverse myelit.

General background

Clinical aspects of NMO

The clinical features of NMO include transverse myelitis (TM) optic neuritis (ON) and brain lesions (10-12). Typically, TM of NMO affects the cervical and the upper thoracic spinal cord segments. Longitudinal extensive transverse myelitis (LETM) is lesions including three or more vertebral segments. LETM or more limited TM starting in the cervical spine and reaching into the brainstem may lead to respiratory failure and/or persistent intractable hiccups and nausea, both of which are regarded as typical for NMO (10, 13). Some studies have demonstrated that one third of NMO relapsing patients experience a severe TM event that results in respiratory failure and subsequent death (10). Episodes of intractable hiccups and nausea as initial presenting symptom have been reported in 43% of cases (12) of NMO patients seropositive for anti-AQP4/NMO-IgG antibodies (14-16). NMO patients often present with complete TM with tetraplegia or paraplegia and a well- defined symmetric sensory affection, usually accompanied by sphincter dysfunction, pain and paroxysmal tonic spasms of the trunk and the extremities (17). ON usually presents with unilateral or less often bilateral ocular pain with loss of visual function (18). ON and TM associated with NMO are often severe and spontaneous recovery of neurological dysfunction is rare and incomplete (19).

The development of anti-AQP4 antibodies/NMO-IgG tests and their use as a diagnostic tool enabled inclusion of NMO patient series with clinical signs and/or lesions in the CNS outside of the optic nerve or spinal cord, thus demonstrating more complex manifestations (10, 20). Recently, certain cerebral presentations, including posterior reversible encephalopathy (21), hypothalamic dysfunction, i.e. amenorrhea, galactorrhea, diabetes insipidus, hypothyroidism, or hyperphagia (22,23) and cognitive dysfunction (24) have been reported to occur in NMO patients.

A number of studies now suggest that monophasic NMO with simultaneous bilateral ON and TM (classical Devic's syndrome) occurs only in a minority of cases, approximately 10 %, with men and women equally affected (10, 25, 26). A secondary progressive clinical course is rarely (approximately 2%) seen in NMO (27). NMO follows a relapsing course in 80 % of cases and this pattern is more commonly seen in females and i s associated with older age. Attacks of ON, TM or both usually occur sequentially rather than simultaneously and the intervals between attacks of ON and TM can be years or even decades (10, 19, 28). In the majority of patients attacks occur frequently and 55% of patients experience relapse within one year, 78% by three years and 90% by five years (9, 10, 29, 30). Clinical consequences of the relapsing type of NMO in more than 50% of patients include visual acuity less than 20/200, or paraplegia with severe residual deficits (10, 12, 31-33). For NMO in general the five-year survival rate is approximately 90% for patients with monophasic disease, and less than 80% for patients with relapsing disease (10, 20).

• *Diagnostic criteria for NMO*

Since NMO is a severe CNS IDD with a less favourable prognosis than MS and with different treatment approaches, early diagnosis based on robust criteria is critical (13). Three sets of criteria have been proposed (Table 1). In 1999 Wingerchuk et al. described the natural history of NMO in a large group of patients (a total of 78) based on demographic and clinical information as well as cerebrospinal fluid (CSF) and MRI features (12). The majority (48/71) of NMO patients followed a relapsing course. The relapsing course group was associated with female gender and older age at onset. Acute cervical TM with involvement of the brain stem was complicated with respiratory failure and death in 15/48 patients of the relapsing group. Brain MRI examination was performed initially in 28 patients and showed normal findings in 25 and MS–like lesions in three. Of 50 MRof the spinal cord an LETM was observed in 44 patients. These observations led to an early definition of criteria for NMO.

However, the criteria from 1999 had limitations because one of the three absolute requirements for NMO diagnosis was the absence of extra-optic-spinal symptoms or signs within the CNS. Furthermore, a supportive criterion was normal brain MRI or findings which did not meet radiological criteria for MS at disease onset. Thus, based on these criteria the clinical distinction from MS sometimes was impossible, when patients had evidence of clinical disease involving other regions of the CNS or had MS-like lesions or lesions in the brainstem. Also it was impossible to diagnose NMO at an early time point before the occurrence of clinical manifestations with a poor clinical prognosis, such as blindness, tetraplegia and respiratory failure (12). These limitations showed a need to include additional diagnostic criteria. Wingerchuk et al. (2006) proposed revised diagnostic criteria for NMO based on radiologic and clinical evidence in 96 patients (Table 1) (10). The authors found that 15% of patients who otherwise met criteria for NMO experienced neurological symptoms referable to disease elsewhere in the CNS and up to 60% had radiologic dissemination (34). In addition this cohort study supported the previous observations (17) that NMO patients followed a relapsing course in the majority of cases and that NMO was associated with female gender (85%) and with older age. Spinal cord MRI with LETM or TM starting in the cervical spine and reaching into the brainstem was regarded as specific for NMO. When NMO-IgG measurement was included in the final diagnosis 76% diagnostic sensitivity and more than 94% specificity was observed. These criteria remove the absolute restriction on CNS involvement to the optic nerves and spinal cord and facilitate the diagnosis of a spectrum of NMO.

The United States National Multiple Sclerosis Society (NMSS) task force on differential diagnosis of MS recently proposed (35) diagnostic criteria for NMO (Table 1). In comparison with the Wingerchuck et al. 2006 criteria the NMSS criteria required the presence of LETM. The timing of neuroimaging (MRI of the spinal cord) then becomes more important, because LETM may break up and appear in multiple shorter plaques (≤3 vertebral segments) during a remission (36, 37).

The NMSS criteria did not include a broader spectrum of NMO, including incomplete/limited manifestations in association with anti-AQP4 antibodies/NMO-IgG seropositivity. However, the NMSS criteria recognized the diagnosis of some of the brain lesions including the hypothalamus, medulla, and other brainstem areas that may have a high expression of AQP4. Other lesions e.g the transient vasogenic edema lesions recently reported (21) were not accepted. Seropositivity for antinuclear antibodies (ANA) or anti-Sjögren's syndrome A (anti-SSA) and anti-Sjögren's syndrome B (anti-SSB) antibodies did not exclude the diagnosis of NMO provided there was lack of clinical evidence for systemic disease such as systemic lupus erythematosus or Sjögren's syndrome.

Wingerchuk 1999	Wingerchuk 2006	NMSS (2008)
Definite NMO **Absolute criteria:** 1) ON 2) TM 3) No clinical disease outside of the optic nerves and spinal cord **Major Supportive Criteria:** 1) Brain MRI not meeting diagnostic criteria for MS 2) LETM on spinal cord MRI 3) CSF pleocytosis (>50 WBC/mm3) OR > 5 neutrophils/mm3 **Minor Supportive Criteria:** 1) Bilateral ON 2) Severe ON with fixed visual acuity worse than 20/200 in at least one eye 3) Severe, fixed, attack-related weakness (MRC grade ≤2) in one or more limbs All absolute criteria plus 1 of 3 major supportive criteria or 2 of 3 minor	**Definite NMO** **Absolute criteria:** 1) ON 2) TM **At least two of three supportive criteria:** 1) Initial brain MRI not meeting diagnostic criteria for MS (McDonald dissemination in space criteria) 2) LETM on spinal cord MRI 3) Anti-AQP4 antibody/NMO-IgG seropositive status **The spectrum of NMO:** 1) Idiopathic single or recurrent events of LETM on MRI 2) ON recurrent or simultaneous bilateral 3) Asian OSMS 4) ON or LETM associated with systemic autoimmune 5) ON or TM associated with brain lesions typical of NMO (hypothalamic, corpus callosal, periventricular, or brainstem)	**Definite NMO** **Absolute criteria:** 1) ON 2) TM MRI T2 hyperintense >3 vertebral segments and T1 hypointense during myelitis Sarcoidosis, vasculitis or SLE (clinically manifest) exclude diagnosis of NMO **At least one of two supportive criteria:** 1)Initial brain MRI not meeting diagnostic criteria for MS (McDonald dissemination in space criteria) 2) Anti-AQP4 antibody/NMO-IgG seropositive status **Do not include the spectrum of NMO**

Table 1 A comparison of the proposed diagnostic criteria for NMO: Wingerchuk 1999, 2006 and NMSS 2008. Reproduced from Asgari et al. (Acta Neurol. Scand 2010) with permission from the publisher.

Clinical variations of NMO: The NMO spectrum

The detection of anti-AQP4 antibodies/NMO-IgG has been used as a supportive criterion in the diagnosis of the definitive form of NMO (10) as described above. The determination of anti-AQP4 antibodies/NMO-IgG is particularly important in the diagnosis of the NMO spectrum, which includes the following clinical settings:

1) Incomplete/ limited forms of NMO
a) Single or recurrent LETM or/and recurrent TM
b) Recurrent or simultaneous bilateral ON
2) LETM or ON with seropositive findings for NMO-IgG associated with :
a) Asian optic-spinal MS (OSMS)
b) Brain MRI lesions
c) Systemic autoimmune disorders

LETM: A LETM gives a high suspicion of NMO and may occur as single or recurrent events. NMO-IgG is supportive in these cases. NMO-IgG seropositivity at the initial presentation of LETM predicted relapse of myelitis or development of optic neuritis in 11/29 (38%) patients (17).

Recurrent or simultaneous bilateral ON: The diagnosis of NMO should be considered in patients who present with a single or recurrent ON especially in cases of severe ON with incomplete visual recovery, bilateral simultaneous ON or sequential ON in rapid succession (30). A follow-up study investigated 72 patients with recurrent ON (≥2 sequential events of ON) over a period of ten years. Five years from their first episode of ON 12 % of these patients developed NMO and 14% developed MS. The risk of converting to MS continued to increase beyond five years; the risk of conversion to NMO seemed to plateau at that point (38). Twenty per cent of patients with recurrent ON were positive for NMO-IgG in both a French (39) and a US series (18). Positive anti-AQP4 antibody was reported in 10% of Japanese patients who had their first–ever ON (40).

OSMS and NMO: Demographic and clinical characteristics of OSMS in Japan and relapsing NMO in the Western hemisphere are sufficiently similar to have suggested that the two entities are identical (12,41, 42). Nonetheless, the relationship between OSMS and Western NMO is complicated and it is presently unclear whether OSMS and NMO are two different entities or not. The diagnostic criteria for OSMS differs as e.g. a LETM in the Western Hemisphere suggests NMO, but in Japan is indicative of classical MS. Furthermore, clinical or radiological involvement of brain lesions other than the optic nerve excludes a diagnosis of NMO in Asian countries, which is not the case for Western NMO (10, 35). Approximately 60% of Japanese patients with OSMS were positive for anti-AQP4 antibodies/NMO-IgG (43). Further studies are needed to characterize differences and similarities between NMO and OSMS.

Brain MRI lesions: LETM or ON may be associated with brain lesions shown on MRI in the hypothalamic, corpus callosal, periventricular or brainstem areas (20). These lesions are now accepted to be compatible with NMO provided other characteristics of NMO or NMO-IgG are present (44). Pittock et al (2006) investigated brain abnormalities in 60 patients with NMO, of which68 % were NMO-IgG positive. Brain abnormalities as shown by MRI were observed in 60% of the patients in serial studies over several years. Ten % of the 60 total study patients had MS–like lesions (five seropositive), five additional patients (8%), three children, had diencephalic, brainstem or cerebral lesions atypical for MS, and all were positive for NMO-IgG.

Systemic autoimmune disorders: LETM and/or ON may occur in patients with systemic autoimmune diseases such as SS and SLE (34). The presence of anti-AQP4 antibodies/NMO-IgG together with LETM or ON qualifies for the diagnosis of NMO spectrum. The co-existence between NMO and systemic autoimmune diseases will be discussed in further detail below.

The seronegative NMO group

NMO patients negative for anti-AQP4 antibodies/NMO-IgG constitute a separate diagnostic challenge even though substantial evidence may be present including clinical and neuroimaging data. Especially the seronegative NMO cases different from the classical NMO type may be difficult to diagnose due to lack of a diagnostic reference standard (10). Determination of anti-AQP4-antibodies in CSF is an interesting diagnostic possibility which deserves further evaluation, but has only been investigated in case reports (45). Seronegative NMO may be explained by the following reasons: a) true negativity, i.e. pathogenic factors other than antibodies are involved, b) differences in assay performance, c) testing while the patient receives immunosuppressive therapies (46), d) antibodies from the anti-AQP4 antibodies/ NMO-IgG seronegative patient may be targeting antigens other than those in the diagnostic antibody assay (47). Interestingly, an in vitro study (48) showed that serum from seronegative NMO patients had a pronounced cytotoxic effect on astrocytes as compared to sera from MS patients and healthy controls.

• *The NMO-IgG and anti-AQP4 antibody assays*

The recognition of serum anti-AQP4 antibodies/NMO-IgG and their role in NMO was first made by Lennon et al (2004), who developed an indirect immunofluorescence assay based on a specific morphological antibody reactivity to mouse CNS structures called NMO-IgG. Sensitivity and specificity of the assay for NMO were reported to be 73% and 91%, respectively. A semiquantitative assay principle was also used by Takahashi et al (2007) and Matsuoka et al. (2008) who employed reactivity towards a green flourescent protein fused with AQP4 (GFP-AQP4) fusion protein and induced into the human cell line HEK-293T (49). Thus, this assay measured the presence of anti-AQP4 antibodies. The sensitivity and specificity of this anti-AQP4 antibody assay were similar to the NMO-IgG assay. In addition, a radioimmuno-precipitation assay has been developed (50) based on ^{35}S

methionine-labelled recombinant AQP4 for detection. For this assay the authors observed a sensitivity of 63% and a specificity of 98%. Also a fluorescence immunoprecipitation assay for AQP4 antibodies has been developed (43), which for NMO patients showed both a high sensitivity and specificity. Furthermore, it was shown that the AQP4 antibodies were predominantly of the IgG1 isotype. Lastly, an enzyme-linked immunosorbent assay (ELISA) has been developed with recombinant rat AQP4 as the antigen to detect serum anti-AQP4 antibodies (26). Anti-AQP4-antibodies as detected by ELISA measured in the same patients indicated a large but not complete overlap with NMO-IgG.
A number of precautions may be taken. The serum antibodies called NMO-IgG may be directed towards different antigenic structures in the mouse CNS. The assays for anti-AQP4 antibodies may detect antibodies towards different antigens in the recombinant rat or human AQP4. Possibly, the differences in sensitivity and specificity of published anti-AQP4 antibodies assays may be due to reactivity towards different AQP4 isoforms. A recent study (51) of human serum reported an improvement from 70% to 97% sensitivity for NMO-IgG using an assay with M-23-expressing AQP4 transfected cells instead of M1-expressing cells. This study suggests the conformational epitopes of M-23 AQP4 as primary targets of serum anti-AQP4 antibodies/NMO-IgG. However, also the organisation of AQP4 in ortogonal array particles (OAPs) may be of importance (51) and the presentation of AQP4 in astrocyte derived cell lines instead of other transformed cell lines such as HEK293 could improve assay performance.
In conclusion, several different immunoassays based on various different immunological techniques for the detection of serum anti-AQP4 antibodies/NMO-IgG have been introduced/ developed in NMO patients and their sensitivities vary broadly, whereas specificities are uniformly high.

- *Genetic epidemiological aspects of NMO*
 Epidemiological studies

Reports of the prevalence of NMO in different ethnic groups suggest that the disease occurs more often in populations of African, East-Asian and Latin American descent than in other groups. The epidemiological studies of NMO are summarized in Table 2. However, these studies were carried out in small populations mostly based on cases from tertiary hospitals with the inherent risk of bias. Only the study by Cabre et al (2001) (52) was population based. Only the studies by Nakashima et al. (2006) (43) and Matsuoka et al (2008) (26) included NMO-IgG measurements, measured by indirect immunofluorescence and cell-based GFP-AQP4 fusion protein assay, respectively, in the diagnostic work-up, whereas the other studies did not use anti-AQP4 antibodies/NMO-IgG measurement.

 Family studies

NMO cases typically appear sporadically, but a few familial NMO cases were reported among identical twins (53) and female family members (54). A case has been reported of a Caucasian mother-daughter pair, who developed NMO at different stages of life (55) which could indicate a genetic susceptibility to NMO. Recently reported (56), familial occurrence of NMO was observed to be more common than expected based on analysis of multiplex families with NMO. In the same study was observed familiar occurrence of other autoimmune diseases together with NMO, suggesting a common autoimmune background.

Country	N	N, MS****	OSMS ***	NMO*/**	Setting
Western Australian	842 (IDD)	703	3.7 %	1.3 %*	Tertiary Hospital
Mexico	424 (MS)	390		8 %*	Tertiary Hospital
Martinique	79 (MS)	62		21.5 %*	Population based
Japan 1 (42)	35 (CMS)	13	54 %	34 %**	Tertiary Hospital
Japan 2	113 (CMS)	54	42.5 %	13.3 %**	Tertiary Hospital

Table 2 Epidemiological studies on neuromyelitis optica (NMO) with selected data. CMS = Clinically definite MS; IDD = Inflammatory demyelinating disease; MS = multiple sclerosis; N= Total population; OSMS = Optic-spinal form of MS.
* Diagnostic criteria for NMO, Wingerchuk et al. 1999; ** Wingerchuk et al. 2006;*** Diagnostic criteria for OSMS, Kira, 2003; ****Diagnostic criteria for MS, Poser et al. 1983. Reproduced from Asgari et al. (Acta Neurol. Scand 2010) with permission from the publisher.

 Population genetics and HLA

Epidemiological studies have suggested ethnicity-based differences of NMO. The genetic basis for the suggested high prevalence of NMO in non-Caucasian populations is not known, but HLA is an obvious candidate. HLA associations in MS include interaction between several different HLA- regions and include both HLA class I and class II antigens. MS studies indicate that expression of some HLA haplotypes are associated with increased risk and other haplotypes with a protective effect on the disease (57, 58). The HLA-DRB1*1501 allele is the strongest genetic susceptibility allele for MS in northern European populations (57, 58).
Studies in Japan have analysed the clinical and genetic features of optic-spinal form of multiple sclerosis (OSMS) which is comparable to NMO. In that population conventional MS was associated with the HLA-DRB1*1501 allele (59), as also seen in Caucasian patients, whereas OSMS/NMO was associated with the HLA DPB1* 0501 allele (29, 60). This allele is at the same time the most common DPB1 allele in the Japanese, expressed by about 60% of the general population (29).
The few studies of HLA in NMO indicate that expression of the HLA allele DRB1*0301 is associated with increased risk of NMO (61-63). HLA- investigations in 42 French Caucasian NMO patients (24 seropositive for NMO-IgG) compared with 310 healthy controls and 161 patients with MS showed that HLADRB1*03 was associated with NMO-IgG seropositivitye (OR 3.08; 95% CI1.52–6.27, p<0.01) (63).

- **Immunological aspects of NMO based on human and experimental studies**

 Aquaporins in CNS

The presence of antibodies with specificity for AQP4 in NMO has accelerated interest in the nature of AQP4 itself. AQPs provide the major route for water movement across cell membranes in many cell types (64, 65). In mammals there are at least 13 classes or isoforms of aquaporins (AQP0– AQP12). Of these at least 4 isoforms (AQP1, AQP4, AQP9, and AQP11) have been identified in the CNS, but the functional role of the more recently discovered

isoforms (AQP9 and AQP11) remains to be established (66) Recent studies indicate that AQP9 is implicated in brain energy metabolism (64, 67). AQP11 does not seem to transport water or any other substrate so far tested (68), but a recent study showed that purified AQP11 protein reconstituted into liposomes increased their permeability for lipids, suggesting a role for lipid transport (69). AQP1 is localized in the choroid plexus and cells lining the ventricles and has a role in CSF formation (70).

AQP4 is by far the most predominant AQP in the CNS. AQP4 is involved in water homeostasis and extracellular osmotic pressure in brain parenchyma (70-73). AQP4 is densely localized in the astrocytic foot processes which underlie the pia mater and the microvessels to form the glia limitans of the blood brain barrier (BBB) (74) and AQP4 is abundant in the grey matter of the spinal cord, the periventricular area and the periaqueductal areas (70-73). Interestingly, AQP4 is also present in osmosensory areas such as the supraoptic nucleus (71). Outside the CNS AQP4 is located in basolateral plasma membranes of epithelia in the kidney collecting ducts, airways, parietal cells of the stomach, skeletal muscle sarcolemma and colon (75).

AQP4 variants

The human AQP4 gene is mapped to chromosome 18 at the junction of q11.2 and q12.1. It is composed of five exons encoding 22, 127, 55, 27 and 92 amino acids and separated by introns of .7, 0.8, 0.3 and 5.2 kb (75-77). The protein monomers consist of six membrane-spanning α-helices and two pore helices that determine the channel's selectivity for water molecules (72, 78). The protein is expressed in two isoforms: M1-AQP4 and M23-AQP4 (32 and 30 kDa in length) generated from alternative transcripts and different translation-initiating methionines. M23 (the shorter form of AQP4) forms higher order assemblies within the plasma cell membrane, termed orthogonal arrays of particles (OAPs), which are nearly immobile, whereas M1 exists as individual tetramers that do not form OAPs (79). It has been shown that M23-AQP4 is stabilized by hydrophobic tetramer-tetramer interactions involving N-terminus residues, and that absence of OAPs in AQP4-M1 results from non-selective blocking of this interaction by residues just upstream from methionine-23 (80). The functional consequences are not known exactly, but this assembly might enhance water permeability or lead to increased plasma membrane stability (81). Recently it has been shown that concentration-dependent binding of NMO-IgG to M1 and M23 expressed affinity variations, but with consistently higher affinity in the binding to M23, preferentially assembled in OAPs (80).

AQP4 polymorphisms have been detected in humans (78). Twenty four AQP4 variants were identified in 188 healthy individuals including 47 African Americans, 47 Caucasians, 47 Chinese and 47 Mexican Americans. Four novel single-nucleotide polymorphisms (I128T, D184E, I205L and M224T) were found, all characterized by reduced water permeability. Distribution of AQP4 variants differed between ethnic groups and the authors suggested that ethnicity-based differences in outcome after brain injury could be based on allelic differences in variant AQP4 genes.

Anti-AQP4 antibodies/NMO-IgG pathogenicity in vitro

In-vitro studies have shown that NMO-IgG binds selectively to AQP4 in astrocytic foot processes of normal mouse CNS tissues (82). Studies in AQP4 knockout mice and AQP4-transfected cell lines supported that AQP4 is a target structure in NMO-IgG-positive patients (82). Recent in vitro studies have addressed the underlying molecular mechanisms of the binding of NMO-IgG to astrocytic AQP4. One study investigated the role of NMO-IgG on BBB function (83). The authors demonstrated that NMO-IgG binding to astrocytes altered AQP4 polarized expression and this process increased permeability of the astrocyte/endothelial barrier. This finding may have implications for the BBB function in NMO. In another study NMO-IgG binding to AQP4 in the presence of complement led to astrocyte membrane damage, accompanied by down regulation of AQP4 as well as down regulation of the excitatory amino acid transporter 2 (EAAT2) also called the glutamate transporter, suggesting disruption of glutamate homeostasis. Kinoshita et al. (2009) have recently confirmed that human anti-AQP4 antibody-positive sera induced necrosis of rat astrocytes in vitro. The cytotoxicity of anti-AQP4 antibodies was observed only in the presence of complement, and immunocytochemical analysis revealed positive staining of human IgG and C5b-9 on astrocytes incubated with sera from patients with NMO (Fig 2).

It may be speculated that anti-AQP4 antibodies/NMO-IgG in the presence of complement lead to CNS inflammation and demyelination. Based on the data presented above, it seems likely that anti- AQP4 antibody/NMO-IgG cause astrocyte injury and is pathogenic in the development of NMO. The anti-AQP4 antibodies/NMO-IgG antibody reactivity was restricted to the CNS as anti-AQP4 antibodies/NMO-IgG did not bind to other peripheral neuronal elements in tissues like liver, kidney, and stomach. It is unclear why anti-AQP4 antibodies/NMO-IgG should spare peripheral organs that express AQP4, such as the kidney and striated muscle. An explanation could be differences in antigen (AQP4) densities. However, a recent paper (84) offers an alternative explanation: anti- AQP4 antibodies/NMO-IgG recognize a conformational epitope on M23-AQP4 assembled in OAPs, but not the native AQP4 protein or M1-AQP4 transfected cells. Thus, differences in tissue distribution of the two AQP4 isoforms could explain the apparent CNS-selectivity of anti-AQP4 antibodies/NMO-IgG pathogenicity.

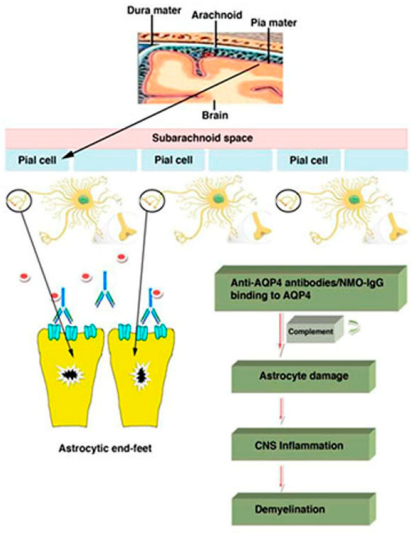

Figure 2
The supposed cytotoxic effects of anti-AQP4/NMO-IgG with activation of complement on astrocyte feet processes at the blood brain barrier. Reproduced from Asgari et al. Acta Neurol. Scand 2011 with permission from the publisher.

Immunopathology of NMO

The histopathology of NMO lesions is characterized by inflammation, demyelination, axonal loss and severe necrosis, which follow the restricted topographic distribution of the lesions. The inflammatory infiltrates in the active lesions are characterized by prominent infiltration by macrophages and granulocytes (eosinophils and neutrophils), and perivascular deposits of immunoglobulin with signs of complement activation, contrary to what is found in MS lesions (33, 42, 85).

The distribution of immune complexes in the tissues corresponds to the normal expression of AQP4 at the end feet of astrocytes (15, 42-44). Distinct areas of AQP4 loss at the astrocyte end feet were observed at the sites of perivascular Ig and complement activation (86, 87). This observation is in contrast to lesions in multiple sclerosis, where AQP4 immunoreactivity is increased.

Lucchinetti et al. (2002) investigated the necrotizing demyelination seen in the spinal cord and optic nerves in NMO. They examined eighty-two lesions from nine autopsies of NMO patients and found an abundance of IgM in immune complexes deposited around penetrating vessels in the lesions. The presence of IgM could be related to the increased density of AQP4 in NMO-prone areas of the CNS. These findings are not clearly compatible with the lack of AQP4 expression in NMO lesions observed in other reports, but hypothetically IgM antibodies may remove or mask the AQP4 at the lesions. It should be noted that the antigen specificity of these IgM antibodies is unknown and may represent anything from rheumatoid factor-like antibodies to non-specific antibody trapped in immune complexes.

Demyelination of CNS axons is now accepted as a major cause of neurological disability in IDD (88). Endogenous repair mechanisms such as remyelination contribute to axonal protection (42). Remyelination has been described as a frequent phenomenon in acute or early MS lesions (89), but signs of remyelination in the NMO-lesions are rare. Chronic NMO lesions are characterized by astrogliosis, cavitation and atrophy (89). Thus, lack of myelin repair appears to be a prominent immunopathogenic mechanism in NMO.

In summary the distinctive features in NMO include severe necrosis which is rarely seen in MS lesions, with prominent infiltration by eosinophils and neutrophils, hyalinization of blood vessels, deposits of complement and IgM as well as IgG, and AQP4 loss. The immunopathology of NMO lesions supports the concept that autoantibodies against AQP4 are involved in the pathogenesis of NMO.

NMO and autoimmunity

o Anti-AQP4 antibodies/NMO-IgG pathogenicity in vivo

Autoimmune diseases are caused by a complex combination of genetic predisposition, environmental assaults such as infection or chemical exposure and defects in immune regulation (89). It may be difficult to prove that a given disease is autoimmune in nature and to identify the autoimmune mechanisms and molecular targets of the disease process. Only few generally accepted autoimmune diseases actually fulfill the four criteria (89):

1) The disease should be associated with humoral or cell mediated autoimmunity

2) The autoantigen should be identified

3) The disease should be transferred by pathogenic antibodies or T-cells

4) The disease should be experimentally induced by presentation of an autoantigen from the target organ.

Criterion 1: Anti-AQP4 antibodies/NMO-IgG was described as a diagnostic marker found in the serum of the majority of patients with NMO and not in patients with other inflammatory CNS IDD such as MS (6, 8). NMO-IgG has been found to be highly specific (85–99%) and reasonably sensitive (58–76%) for NMO (6, 43). Recent papers have documented correlations between anti-AQP4 antibody titres and the severity of the clinical course. Anti-AQP4 antibody titers were overall higher in patients during relapse than during remission and diminished anti-AQP4 antibody levels were seen during immunosuppressive therapies (9, 90).

Criterion 2: NMO-IgG binds selectively to AQP4 in astrocytic foot processes of normal mouse CNS tissues (82). Several other studies have confirmed the specific binding in vitro of anti-AQP4 antibodies/NMO-IgG to AQP4 (83, 91, 92). Recently, intrathecal production of AQP4 antibody was shown by the isolation of local plasma cells and synthesis of antibodies followed by demonstration of antibody specificity and its pathogenic potential (93). Interestingly, the intrathecal humoral immune response against AQP4 was obtained at the onset of clinical disease.

Criterion 3: An obvious human model for transfer of disease would be the transmission of NMO from the mother to the newborn infant. However, no case of transplacental transmission of anti- AQP4 antibodies/NMO-IgG from an afflicted mother to the fetus/infant has to our knowledge been reported. Experimentally, in vivo transfer of inflammatory activity into MBP-specific experimental autoimmune encephalomyelitis (EAE)-primed rats by purified IgG from NMO patient sera has been reported, using MS patient serum as control (94). The anti-AQP4 antibody induced loss of AQP4- positive astrocytes and exacerbated EAE-like pathology. A limit to this experimental design may be restriction by the BBB of access to CNS of IgG and the possibly limited amounts of complement and other pathogenic factors in the CSF. Recently, Bradl et al. (2009) demonstrated transfer of inflammatory activity with NMO-like characteristics into already established EAE with IgG from AQP-4 antibody–positive NMO patient. These authors demonstrated a NMO-like immunopathology with astrocyte necrosis, loss of AQP4, and immunoglobulin and complement deposition. No pathological changes were found in peripheral organs such as the kidney and muscle (95). Bennett et al. (2009) demonstrated that intrathecal anti-AQP4 antibodies/NMO-IgG generated from one NMO patient could induce NMO-like changes in conjunction with EAE (93). Recently it has been demonstrated that intra-cerebral injection of IgG from an NMO patient together with complement induced NMO-like lesions in mice, whereas no reactions occurred without complement or with IgG from controls or injection into AQP-4 null mice (96). This model was supplemented by a study in athymic nude mice which showed identical results indicating that the NMO-like disease process occurred independently of T cells (97). However, a recent thorough study demonstrated in Lewis rats the role of AQP4-specific T cells in NMO-like lesions aided by anti-AQP4 antibodies (98).

Criterion 4: Miyamoto et al. (2009) analysed AQP4 expression in the CNS in mice with EAE. They observed an up-regulation of AQP4 and proposed that AQP4 was involved in the development of inflammation in the acute phase of EAE. Although more direct evidence such as active anti-AQP4 immunity has been difficult to produce in animal models, autoimmunity to other CNS antigens has been shown to induce NMO-like disease. A transgenic mouse model with a T cell receptor (TCR) specifically reactive to a myelin oligodendrocyte glycoprotein (MOG) peptide was demonstrated

to lead to monosymptomatic (99). This model was developed further by crossing the MOG-TCR mouse with an IgG anti-MOG heavy chain knock-in mouse (100). The majority of the double-transgenic mice developed lesions of the optic nerve as well as inflammation of the spinal cord. This model thus showed pathological changes which resemble human NMO, but related to the antigen MOG and not to AQP4. Lastly, it has been recently shown that AQP4 knock-out mice with EAE produced by MOG-protein immunization showed a significantly attenuated disease process as compared to wild-type mice suggesting that AQP4 is a determinant in autoimmune inflammatory disease in the CNS (101).
In summary, while active induction of NMO by antigenic stimulation in a naïve animal has not been demonstrated, induction of NMO-like pathology has been achieved in EAE models (94, 95, 99) and by intra-cerebral administration of antibody to naive mice supporting a role for anti-AQP4 antibody production in the disease process.

o Indirect evidence for autoimmunity in NMO

The disease course should be prevented or improved by immunosuppressive therapy Current therapies for NMO include glucocorticoids, azathioprine, and plasmapheresis. Intravenous immunoglobulin treatment has been reported to improve the neurologic outcome for patients who have NMO or NMO spectrum disease (102-104). Also, other immunosuppressive therapies such as cyclophosphamide, mitoxantrone, cyclosporine, methotrexate and mycophenolate mofetil have been applied. However, patients commonly relapse on these treatments (25, 90, 105). Rituximab is an IgG1 monoclonal human/murine chimeric antibody directed against the CD20 antigen, which is expressed on the surface of nearly all mature B lymphocytes (106). Small studies with limited numbers of patients have been performed in NMO patients with refractory disease (90, 105). In one study, seven of 8 patients responded to the Rituximab therapy with improvement in pyramidal, sensory, visual, bowel, and bladder nervous function (102). In another study treatment with Rituximab reduced the frequency of attacks, with subsequent stabilization or improvement in disability in 20 of 25 patients (107). The results of these few studies support the view that Rituximab is a promising treatment in NMO. However, this treatment principle for NMO is still at an early investigational stage and randomized controlled studies are needed.

o Clinical co – existence between NMO and other autoimmune diseases

The B cells have an important role in regulating many aspects of immune reactivity, as well as the capacity to differentiate into autoantibody-producing cells. Defects in the regulation of B cell immunity have suggestively been associated with antibody-mediated autoimmune diseases, perhaps as a result of genetic abnormalities that directly regulate B cells (108). Antinuclear antibodies, anti- SSA and anti-SSB antibodies and in particular anti-MOG antibodies have been found in NMO patient sera as well as in CSF (109) suggesting a general susceptibility to antibody-mediated autoimmune disease. Both organ-specific autoimmune diseases such as myasthenia gravis, autoimmune thyroiditis (46, 110, 111) and non-organ-specific autoimmune diseases such as SLE and SS (34, 112, 113) have been associated with NMO. Furthermore, both types of autoimmune diseases may coexist in the same patient, either sequentially or concurrently, sustained by the presence of autoantibodies directed against the corresponding autoantigens (113, 114). In a literature search for cases of LETM secondary to systemic lupus erythematosus (SLE), twenty-two such patients were found. LETM was the first manifestation of SLE in five patients. However, the authors did not consider the diagnosis of NMO (115). Recently, Pittock et al. (2008) investigated clinical and serological associations between NMO spectrum and a range of systemic autoimmune diseases, mostly SLE and SS. NMO-IgG was detected in most sera of patients with concomitant NMO or NMO spectrum concomitant with SS or SLE, but NMO-IgG was not found in sera of SLE or SS patients with no manifestations of NMO such as LETM or ON. This association is an indication of coexisting NMO rather than the presence of a vasculopathy or other complications of systemic autoimmune disease. Another recent study (111) investigated the prevalence of myasthenia gravis in 177 patients with NMO and 250 control patients (173 healthy; 77 MS). The frequency of muscle acetylcholine receptor (AChR) antibodies was 11% in NMO. There was a 2% prevalence of clinical myasthenia gravis in NMO patients (vs. at most 0.02% in the general population.

Current status

The clinical spectrum of NMO is by now reasonably well-described. NMO was previously considered to be confined to the optic nerve and spinal cord. Discovery of NMO-IgG led to the recognition of NMO patients with clinical signs and/or lesions in the CNS outside of the optic nerve and spinal cord. Brain abnormalities have in limited studies been detected by MRI- and compared with clinical outcome. Interestingly, all studies have been based on ON and TM as the initial symptoms. Other clinical manifestations may also signify the onset of the pathological process in NMO, but this possibility has as yet not been investigated. Further clinical and serological investigations are needed to evaluate the specificity of anti-AQP4 antibodies/NMO-IgG for specific syndromes in NMO.
Different sets of criteria for NMO have been introduced during the last decade. However, these criteria need validation in different populations and in large, preferably multi-center investigations. Limited knowledge still exists of the prevalence of NMO in Caucasians. Population-based studies in particular in Caucasian populations with clinical characterization and standardised antibody assays are therefore needed before it is possible to conclude that different ethnic groups have different epidemiology of NMO.
The few studies of HLA in NMO indicate that HLA associations for NMO seem to be different from the associations reported for MS. The immunological and genetic background for the association of NMO with other autoimmune diseases has not been investigated.
Immunopathological evidence from NMO lesions in human studies and animal models suggests that anti-AQP4 antibodies/NMO-IgG are involved in the pathogenesis of NMO. A considerable amount of recent experimental evidence has been presented for transfer of disease activity. However, there is not convincing evidence for pathogenicity of plasma-AQP4 antibodies in unprimed animals and all known animal models depend on a breach of BBB in access to CNS of IgG. It is unclear whether circulating anti-AQP4 antibodies/NMO-IgG cross the BBB and the cerebrospinal fluid (CSF)- parenchymal barrier to get access to enter the CNS. A global evaluation to determine whether the distribution of NMO-like histopathology in the CNS corresponds to the normal expression of AQP4 antigen has so far not been performed. Such an evaluation would increase the understanding of the role of anti-AQP4 antibodies/NMO-IgG in NMO pathogene-

sis. Furthermore, other immune mechanisms may be concurrently active in NMO, notably T cells.

Aims of the thesis

Epidemiological and clinical study:
1- To estimate the incidence and prevalence of NMO in a predominantly Caucasian population and to perform a clinical characterization of the NMO patients in this population.
2- To investigate immunogenetic and autoimmune aspects of NMO including HLA.

Experimental study:
3- To induce an NMO-like disease activity by transfer of purified IgG from AQP-4 antibody–positive NMO patient into mice.
4- To characterize the effects of human anti-AQP4 antibodies/NMO-IgG in the CNS in an animal model for NMO.

MATERIALS AND METHODS

- Epidemiological and clinical methods

The study was designed as a population-based, historical cohort study with clinical and questionnaire follow-up in those patients available. It took place in a well-defined geographical region, The Region of Southern Denmark, which comprises a population of 952 000 adult inhabitants, corresponding to approximately 1/5 of the Danish population (Fig 3).

Figure 3
The Region of southern Denmark

o Patients and sources of data

Sources of data: Information about all patients > 18years of age who acquired a diagnosis of multiple sclerosis (MS), neuromyelitis optica (NMO), optic neuritis (ON), transverse myelitis (TM) (WHO ICD-10 codes: G35- H46.9- G 37.3- G360-) in the period January 1, 1998 - December 31, 2008 were obtained from the four Neurology and three Ophthalmology departments in the Region of Southern Denmark. In addition, all patients from a separate register of MS patients treated with biological therapy (natalizumab) from the neurological departments were included.
All Danish citizens are registered in the Danish Civil Registration System and identified by a unique personal identification number (CPR-no.) and information about patients included this CPR- no. This facilitated a cross-check of data from the departments with the Danish National Patient Registry (DNPR)(116). The DNPR includes discharge diagnosis from hospital visits as well as outpatient contacts in the Danish health care system. The registry is kept by the National Board of Health, Denmark. In addition to this information the Research Service of the Board provided updated information on status (dead/alive/emigrated), addresses and whether a person were registered with "research protection".

o Inclusion criteria and exclusion criteria

Patients were included in the study base if they fulfilled the following inclusion criteria

1- Episodes of ON and/or TM
2- An initial brain MRI (obtained within the first year of the onset of symptoms) that did not meet diagnostic criteria for MS at disease onset (McDonald dissemination in space criteria) (117, 118)
3- The diagnoses should be established during the investigated decade.
Patients who had ON or/and TM on the basis of vascular, infectious, metabolic, neoplastic, toxic causes were excluded.

o Web-based questionnaire and clinical database

All patients were sent an invitation to take part in the study and were asked to fill in a questionnaire, designed for multiple sclerosis (MS), transverse myelitis (TM) and optic-neuritis (ON), respectively. The patients had the possibility to complete the questionnaire on the Internet entering a given code, or fill in a paper version to be returned by post.
A specially designed neurological filing system in the form of a web-based database was used to integrate all data from questionnaires, medical records and neurological examination. This filing system was designed by NA with technical support by stud. scient. Peter Haagerup.

o Review of the medical records

The medical records for all patients were obtained from the respective departments, by access to either electronic or paper based patient files. Medical information retrieved included: demographic characteristics, dates of symptom onset, clinical presentation, disease duration, dates of CNS MRIs, CSF results, dates of the measurement of visual evoked potentials (VEP), treatments and Expanded Disability Status Scale (EDSS) score. The EDSS were used as a clinical rating scale to classify the degree of neurological dysfunction. EDSS was retrieved from the medical records and in some cases from own examination. Clinical neurological examination was performed when the patient had experienced an acute episode since the last hospital visit or reported an altered clinical status. Furthermore, blood samples were taken.

o The diagnostic process

NMO diagnosis was based on Wingerchuk et al 2006. The diagnostic process consisted of three independent parts (Fig 4):
1- The clinical process:
a) i) The data from patient's files and the questionnaire were integrated in the database.
b) i) The patients underwent a clinical, neurological examination.
ii) Supplementary MRI
was ordered if a clinical relapse was suspected since the last MRI.
iii) The ph.d.-student (neurologist) was blinded to anti-AQP4 results and the re-evaluated and supplementary MRIs of the CNS at this stage.

2- The imaging process:

3- a) The previous MRI's at disease onset and subsequent MRIs were re-evaluated by the neuroradiologist.
b) Supplementary MRI was performed if pre-study MRIs were unavailable. MRI data were reported in a written form by the neuroradiologist.
The neuroradiologist had no prior knowledge of clinical history or results of other investigations.

4- The serological process:
Anti-AQP4 antibodies were determined.
The laboratory staff was blinded to the clinical status of patients when performing the assay. The data were reported in a written form.
In summary, the clinical data and the imaging were prepared to establish the clinical NMO diagnosis. Then the AQP4 antibodies were measured and a final diagnosis of NMO was made.

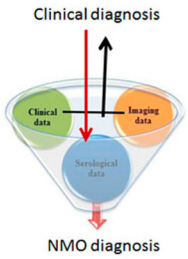

Figure 4
The diagnostic process in the study reported in Article I. First the clinical diagnosis was established based on clinical and radiological data, then the serology was determined and a final diagnosis of NMO was established.

Radiological methods

Since the study was retrospective, different types of MRI scanners were used with a variety of imaging techniques. T2-weighted (T2W), T1-weighted (T2W) images with or without gadolinium (Gd), diffusion-weighted imaging (DWI) and fluid-attenuated inversion recovery (FLAIR) sequences were analysed in MRIs of brain. T2W, T1W with or without gadolinium and short tau inversion recovery (STIR) sequences were analysed in spinal cord imaging. Supplementary MRIs of CNS were performed on a 1.5 Tesla scanner (GE, Paris, France). Pre-study MRIs at disease onset and subsequent MRIs were classified into the three following subgroups: (1) normal, (2) nonspecific lesions, (3) MS-like lesions, i.e. meeting the Barkhof criteria (117) for dissemination in space used in the McDonald criteria (118). Spinal cord MRI was either reported as normal, as abnormal with a smaller lesion not suggestive of NMO or as LETM with high signal on T2- weighted images and if obtained during acute episodes of myelitis with hypointensity on T1- weighted images. CNS atrophy was reported as no atrophy, moderate atrophy or severe atrophy. (119, 120).
The MRI findings were independently re-evaluated manually by one neuroradiologist who was blinded to clinical history and results of other investigations. MRI data were reported in a written form by the neuroradiologist.

Laboratory methods

IgG AQP4 antibodies were measured with a recombinant immunofluorescence assay using HEK293 cells transfected with recombinant human full-length AQP4 gene (Euroimmun, Lubeck, Germany). Patient sera were screened at a 1:10 dilution. Analyses were done in an accredited laboratory at the Department of Clinical Immunology, Odense University Hospital.
Other laboratory methods are described in article II.

o Ethical considerations

The study was approved by The Committee on Biomedical Research Ethics for the Region of Southern Denmark (ref. no. S-20080142) and The Danish Data Protection Agency (ref. no. 2008 – 41-2826). All patients provided oral as well as written informed consent. Clinical data and biological material was registered within the Odense Patient data Explorative Network (the OPEN project) to enable future collaborate projects.

o Statistics

Prevalence was estimated as the number of patients diagnosed with NMO per 100,000 persons in in the total population of the Region South Denmark. Incidence rates were calculated as the number of patients with NMO during the follow-up period divided by the total numbers of person years at risk and reported per 105 person-years. Allelic frequencies were estimated using direct counting method and compared using the chi-square test or Fisher´s exact test probability test when the criteria for the chi-square test were not fulfilled. Odds ratios (OR) were obtained from Woolf´s method. For allelic comparisons, Bonferoni´s method was used as correction for multiple comparisons.

- Experimental study

o Experimental design:

Animal models are an important tool in the elucidation of immune pathogenesis. The presence of anti-AQP4 antibodies in the majority (70-80 %) of NMO patients and histopathological evidence suggest that these antibodies are involved in the pathogenesis of NMO, but do not exclude that there may be forms of NMO which do not only involve autoantibodies, or involve antibodies with specificities other than AQP4 (42, 82). Pathogenicity of anti-AQP4 antibodies/NMO-IgG has been demonstrated by in vivo transfer of purified IgG from an NMO patient leading to an NMO-like condition (93-96). However, it has been difficult to produce NMO by IgG transfer to blood of previously healthy mice and all known animal models have involved a breach of blood–brain barrier (BBB) giving access of IgG to the CNS. Furthermore, a global evaluation of NMO-like histopathology including BBB, cerebrospinal fluid (CSF)-brain/spinal cord barriers, brain and spinal cord has not been done. Such an evaluation is needed to understand the pathogenesis of anti- AQP4 antibodies/NMO-IgG and to determine whether the distribution of NMO-like histopathology in the CNS corresponds to the normal expression of AQP4 antigen.
We decided to investigate whether intrathecal administration (into the cisterna magna) of purified IgG from AQP-4 antibody–positive NMO patient (NMO-IgG) given together with human complement (huC') induces a NMO-like condition in mice (Fig 4). The intrathecal route has previously been used for introduction to CSF of virally-encoded mediators (121, 122). The purpose of the study was to characterize the pathogenic mechanism of anti-AQP4 antibodies/NMO-IgG on the CSF-parenchymal barrier and CNS.

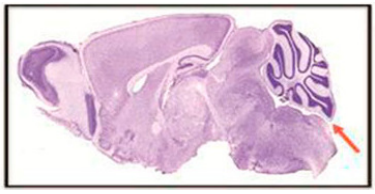

Figure 5
A sagittal section of a mouse brain. Intrathecal injection into the cisterna magna.

Three experiments addressed the aims mentioned above. All experiments were performed in a blinded fashion. The procedures and conditions were the same for all experiments.
1) To determine whether intrathecal administration (into the cisterna magna) of NMO-IgG + huC' leads to NMO-like changes in naive C57BL/6 mice.
Experiment: A total of 10 C57BL/6-mice received NMO-IgG + huC' by intrathecal injection. As negative controls were used 10 C57BL/6 -mice injected with normal human IgG and huC` (normal human IgG + huC`). As additional controls one C57BL/6 -mouse was injected with only huC`, one with NMO-IgG, five with PBS and four were unmanipulated. Mice were sacrificed at Day 7. For practical reasons the mice received identical treatment protocols at three separate times.
2) To determine whether intrathecal administration of NMO-IgG + huC' in C57BL/6-backcrossed T-cell receptor transgenic mice (2D2), which have high frequency of myelin oligodendrocyte glycoprotein-specific T cells, leads to NMO-like disease and to compare the effects with C57BL/6- mice.
Preliminary experiment: Six 2D2-mice received NMO-IgG + huC' by intrathecal injection. As negative control four 2D2-mice were injected with normal human IgG + huC`. For practical
reasons the mice received identical treatment protocols at two separate times. Two 2D2 –mice were injected with PBS and furthermore two unmanipulated 2D2-mice were used as control. All mice were sacrificed on Day 7.

Materials and methods

Human IgG: IgG was obtained from human plasma strongly positive for AQP4-antibody. The plasma originated from a female NMO patient who underwent plasmapheresis. For further details, see Article III.
Human complement: Human complement originated from serum of a pool of healthy blood donors.
Mice: Adult female C57BL/6 mice were purchased from Taconic (Taconic Europe A/S, Ry, Denmark), and MOG-specific TCR transgenic (2D2) mice were originally obtained from Hartmut Wekerle (Max-Planck-Institute of Neurobiology, D-82152 Martinsried, Germany) and were bred in our facility. The mice entered the experiment at the age of 8-12 weeks with weights between 17 and 24 g. The mice were kept according to standard operating procedures of the Biomedical Laboratory, University of Southern Denmark, in accordance with guidelines from the Danish Animal Research Committee (approval number 2009/561-1724). All experiments conformed to Danish guidelines on the ethical use of animals.
Intrathecal injections: See Article III.
Tissue processing: See Article III.
Histology: Histological analysis was performed in a blinded fashion without knowledge of study group allocation of the individual mouse. Histological changes were graded as: + denotes mild changes, ++ denotes moderate changes and +++ denotes marked changes in topographically defined areas including spinal cord, brainstem, cerebellum, midbrain, cortex and periventricular areas.

Histochemistry and Immunohistochemistry: See Article III
Microscopy: See Article III
Animal assessment:The animals were assessed by measurement of whole body weight and gross evaluation of well- being. Assessment of behavioral or motor changes was not part of the study design. The weight of the animals did not show any differences between mice that received NMO-IgG + huC' and controls.

RESULTS

o Epidemiological and clinical study

A total of 477 patient cases were evaluated in the study based on the cross-check of data obtained from the respective clinical departments in the Region of Southern Denmark and the DNPR. This procedure was chosen because samples from DNPR included subjects not residing in the region or who had acquired the relevant diagnosis before the time period. The patient population consisted of 277 MS, 8 NMO, 128 ON and 64 TM patients including 66 MS patients treated with natalizumab. Research protection was registered for 42 patients (9 MS patients, 22 ON patients, 11 TM patients) who were therefore not approached. The patients were subsequently contacted by means of questionnaire with 70% participation. A total of 42 patients did not want to participate in the study (16 MS, 19 ON, 7 TM). The inclusion criteria were not met in 166 MS patients (including 91 patients who had been diagnosed before the inclusion period), 3 NMO patients, 43 ON patients, and 18 patients from the TM group leaving 163 patients (86 MS, 5 NMO, 44 ON, 28 TM) who fulfilled the inclusion criteria. These 163 patients all participated fully in the study except one NMO-patient who died before clinical examination and blood sampling could be carried out. Two NMO patients died after completion of the examinations.
A total of 42 patients qualified for the NMO diagnosis according to the Wingerchuk 2006 criteria (5). All were Caucasians except one patient of African descent. The group consisted of 31 females and 11 males (ratio 2.8:1). Mean age at onset was 35.6 years (range 15–64 years). The yearly incidence rate of NMO in the population was estimated to be 0.4 per 105 person-years (95% CI 0.30- 0.54) and the prevalence was 4.4 per 105 (95% CI 3.1 - 5.7). For further details, see Article I.
The clinical presentation was heterogeneous including TM, longitudinal extensive TM, ON and brainstem syndromes (Fig. 5). All definite NMO patients followed a relapsing course. Follow-up MRI analysis was available for 31 patients. Brainstem lesions occurred in 25 patients at follow-up. MRI-lesions in the medulla oblongata were detected in 18 (58 %) patients. Of those 11 (61 %) had lesions in the area postrema. AQP4-antibody determinations were positive in 72 % of the patients with brainstem lesions.
Based on questionnaire, interview and review of medical records brainstem symptoms were typically polysymptomatic and reversible and included symptoms such as respiratory failure, intractable hiccups and nausea, vomiting, vertigo, diplopia, facial weakness, nystagmus, ataxia and bradycardia and blood pressure fluctuations.

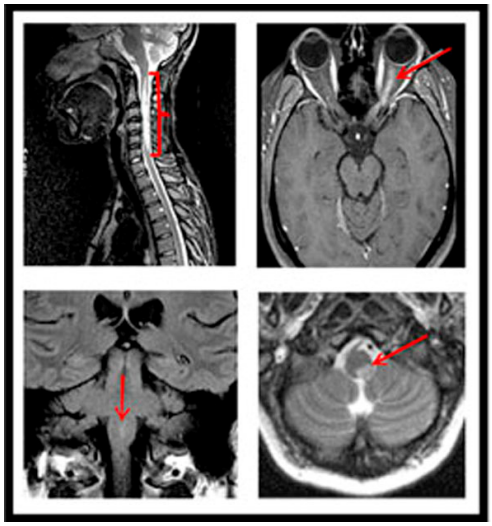

Figure 6
Central nervous system lesions typical of NMO. Representative magnetic resonance imaging (MRI) of patients with NMO, seropositive for anti-aquaporin4 antibodies / NMO-IgG, A) Sagittal T2-weighted MRI of cervical spinal cord showing longitudinally extensive transverse myelitis, B) Axial Gadolinium enhanced T1-weighted image showing enhancing optic neuritis on the left side, C) coronal FLAIR image showing lesion in medulla oblongata D) axial T2-weighted image showing lesion in area postrema. Reproduced from Asgari et al. Acta Neurol. Scand 2011 permission from the publisher.

In the study LETMs were observed in 28 NMO patients. (Article I). Follow-up MRI of spinal cord later showed LETM in additional two patients. In total 23 out of 30 had follow-up MRI of spinal cord, 17 were seropositive. LETMs in 9/23 patients changed into multiple shorter plaques. In the chronic stage spinal cord atrophy at the site of previous inflammation was seen in five patients, after 2-3 year duration of disease. Additionally, seven NMO patients with LETM had severe general atrophy of the spinal cord after 5-10 years duration of disease in four and 3-5 years in two.
Anti-AQP4 antibodies: Anti-AQP4 antibodies were positive in the 26/42 (62 %) of the patients. Antibody positivity was necessary to confirm the diagnosis in 15 cases (36 %), whereas 27 (64 %) were diagnosed solely on clinical criteria. We observed a sensitivity of 62% and a specificity of 100 % for this assay.
Fifty MS patients identified in the study database were evaluated clinically and radiologically verifying the MS diagnosis and were used as disease controls together with 50 healthy controls. None were positive for anti-AQP4 antibodies.

o Clinical immunogenetic study

A higher frequency of a family history (17 %) of inflammatory demyelinating disease (IDD) was found as compared to MS ($p<0.026$). Furthermore, 15 % of NMO patients had other autoimmune disorders and 39 % had family occurrence of autoimmune diseases. The HLA-DRB1*15 and DQB1*06 alleles, which is the strongest genetic susceptibility allele for multiple sclerosis (MS) in northern European populations was increased in MS ($p<0.0027$), but not in NMO patients as compared to controls. The frequency of the HLA-DQB1*0402 allele was higher in NMO patients compared to controls. HLA-DQB1*0402 has been reported to be associated with autoimmune diseases such as primary biliary cirrhosis, type 1 diabetes and juvenile idiopathic arthritis (123-125). Additionally two other genetic markers of autoimmunity, PD-1 and PTPN22 were investigated. No significant association with the PTPN22 1858 T was detected in NMO patients. The PD-1.3A allele was increased in NMO ($p<0.0023$) as compared to controls. For further details, see Article II.

o Experimental study results

Intrathecal administration of human NMO-IgG + huC' produced NMO-like lesions:

Ependymal lining:
Intrathecal injection of NMO-IgG + huC' induced focal disturbance of ependymal lining which was replaced by inflammatory infiltrates with immunopositive CD45, deposition of activated complement (C9neo, a marker of activated complement) and immunoglobulin deposition. A loss of AQP4 expression and reactive astrocytes as indicated by loss of glial fibrillary acidic protein (GFAP) co-localized with leukocyte infiltration and penetrated into the parenchyma. This histopathology was not seen in control mice (See Article III).

Dissemination in CNS:
Intrathecal NMO-IgG+ huC' led to disseminated deposition of immunoglobulin and C9neo, loss of AQP4 and GFAP expression accompanied by inflammation and demyelination. These findings were observed in topographically restricted areas in the cerebellum, brainstem and the parenchyma around periventricular areas including the fourth and lateral ventricles co-localizing with inflammation. (See Article III).

T-cell receptor transgenic mice (2D2):

In order to determine if NMO-like lesions in WT mice were replicated in T-cell receptor transgenic mice (2D2), which have deranged immunity with a high frequency of myelin oligodendrocyte glycoprotein-specific T cells, we performed similar experiments in such mice. Preliminary results showed that NMO-like histopathological changes were markedly increased in spinal cord in 2D2 mice compared to WT mice after 7 days. Such pathology was not seen in 2D2 mice receiving normal human IgG + huC` and mice receiving PBS or unmanipulated (data not shown).

DISCUSSION AND CONCLUSIONS

This Ph.D. thesis provided data on the prevalence and incidence of NMO in a predominantly Caucasian population. A characterization of the natural course of the disease was performed describing the clinical phenotype. It was revealed that LETM tended to occur during the course of NMO and that lesions in the brainstem occurred in a significant proportion of the patients. A goal of this PhD thesis was to investigate to what extent NMO fulfills criteria for an autoimmune disease, with specific clinical, immunogenetic and experimental perspectives. Anti-AQP4 antibodies/NMO- IgG were found in the serum of the majority of NMO patients and a high diagnostic specificity and moderate to low sensitivity of anti-AQP4-antibody determination was observed. A significant proportion of NMO patients had a family history with autoimmune diseases and other inflammatory demyelinating diseases of the CNS and NMO co-existed with other autoimmune diseases. We observed a significantly increased frequency of the PD-1.3A allele, a common susceptibility allele for autoimmune disease. The frequency of the HLA-DQB1*0402 allele which has been reported to be associated with autoimmune diseases was observed to be

increased in NMO patients. Lastly, in experimental studies we observed pathogenicity of anti-AQP4 antibodies /NMO-IgG. Antibody crossed the CSF-parenchymal barrier and produced NMO-like lesions at restricted sites corresponding to expression of AQP4 in the CNS. These data define a novel animal model, which closely mimics human NMO and identifies that once CSF access occurs eg. via circumventricular organs including area postrema, then CSF-brain transit and NMO-like pathology follows.

The epidemiological and clinical study contained some limitations mainly due to data sampling. Data sources were the DNPR and the clinical departments. The number of MS cases was fewer than expected raising issues of incomplete ascertainment. The lower than expected number of MS cases could be due to the broad classification of MS as either a clinically isolated syndrome (CIS), an inflammatory demyelinating disease or as other disorders, whereas the present study selection was solely based on the WHO diagnostic codes for MS. Similar problems have been reported to occur for rheumatological diagnoses (126) and recently for the diagnosis of MS, which was incorrect in a significant proportion of patients (127). Secondly, it proved difficult both for the clinical departments and the DNPR to separate identification of newly diagnosed patients in the study period from patients diagnosed outside of this period, possibly leading to unequal sampling.

A further possible limitation of the study was the selected high-risk MS group who fulfilled the inclusion criteria. Of those who fulfilled the inclusion criteria 35 received natalizumab treatment, which according to general treatment guidelines is given to patients with high disease activity. This strategy may hinder the diagnosis of NMO, but all other things equal increases the prevalence of NMO patients in the MS group, as NMO is expected to be found in the patients with high disease activity.

Another limitation of the present study was the retrospective design of the clinical study. Different types of MRI scanners were used with a variety of imaging techniques and a variety of intervals from onset of clinical manifestations to MRI examination. Also, the clinical evaluation including the EDSS was done by different clinicians. Furthermore, it was not possible to retrieve the initial brain MRI for evaluation in a number of patients and there was a lack of MRI of spinal cord at disease onset in the majority of the patients. Some patients might have been missed by the second inclusion criterion (MRI brain non-diagnostic for MS) as a small proportion of patients with NMO initially had MS-like brain lesions. All MRIs were analysed manually and were reported by a single neuroradiologist (HPBS) and the conclusions were not independently confirmed by another neuroradiologist. However, the participating neuroradiologist was blinded to clinical and antibody status of the patients.

The serological diagnosis by the AQP4 antibody determinations also has some limitations. Sensitivity and specificity of this assay have been reported to be 73% and 91% for NMO (82) and we observed a sensitivity of 62% and a specificity of 100%, respectively. Antibody titers are probably diminished in clinical remission and during immunosuppressive treatment (9, 90), and most of the patients in the present study were in remission or on immunosuppressive treatment. Furthermore, a limitation of this study was the lack of validation of the AQP4-antibody assay. However, the analysis was done in a certified laboratory and performed blinded to diagnosis and clinical and radiological details.

Characterization of the immunogenetic background for NMO was another aim of the study. The relatively speaking small sample size was a limitation of the immunogenetic analyses. An association of the NMO group to the DRB1*0301 allele as compared to healthy controls could not be demonstrated in our study, different from Zéphir et al. (2010) (63) in spite of a nearly similar sample size and HLA DRB1*0301-distribution in the NMO patients. This result may be related to a lower number of healthy controls and MS patients in our study. In the study we used $p < 0.05$ as limit of significance and this decision implies that tendencies were not discussed in detail, a particularly important limitation in small populations as the present. The PD-1 differences were small, as no significant difference was observed in the genotypes, only in the alleles. The allele frequencies do give a valid presentation, particularly considering the small patient group, and we find the report of interest as such investigation has not been reported previously.

Strength of the epidemiological and clinical study was the diagnostic algorithm for NMO, as the clinical diagnosis was established without knowledge of AQP4 antibody results and vice versa, diminishing bias in the study. The review of serological data in a blinded fashion facilitated an analysis of the diagnostic accuracy of anti-AQP4 antibodies/NMO-IgG. Furthermore, all available MRIs were re-evaluated and supplementary MRIs including brain and spinal MRI were taken if missing or if a relapse had occurred since the last MRI, giving a high degree of diagnostic completeness. The prevalence and incidence of NMO were given according to both the diagnostic

criteria from Wingerchuk et al. 2006 as well as the US National Multiple Sclerosis Society (NMSS),2008 (10, 35). Only the Wingerchuk criteria include the limited form of NMO (as part of the "NMO spectrum"). Our data suggest that the limited form of NMO exists in a significant proportion of NMO cases. Even though the study is based on a limited patient population it does represent a valid, even conservative estimate of the prevalence of NMO because of the unselected population-based design, which provided the basis for the incidence and prevalence estimates. The study indicated that NMO is more common in a Caucasian population than earlier believed. As a consequence NMO may be considered a more obvious differential diagnosis than previously thought in diagnostic algorithms for MS as well as for other CNS IDD.

In the present study the diagnosis of NMO could be made purely on clinical grounds in a high proportion (64 %) of cases, based on either the Wingerchuk 2006 criteria or the NMSS 2008 criteria. Similarly, a study from France using the Wingerchuk 2006 criteria found that clinical and MRI-based diagnosis of definite NMO was sufficient in 90 % of 125 NMO patients (31). We observed heterogeneity of clinical disease manifestations similar to the findings of previous studies (10, 31, 128). Interestingly, recent studies report a high frequency of brainstem lesions, suggesting that the brainstem, in particular medulla oblongata and area postrema, are important points of attack in NMO (16, 31, 128-130). Speculatively, the brainstem lesions may be related to the areas with high density of AQP4 expression and lack of blood brain barrier.

Human immunogenetic aspects were studied, investigating HLA and polymorphisms of the T cell associated molecules PTPN22 and PD-1. A strength of the study was the confirmation that the MS group, but not the NMO group is associated to DRB1*1501 and DQB1*0602, as reported previously for other Northern European MS patients (35). The frequency of HLA-DQB1*0402 allele was increased in NMO patients in the present study. Interestingly, HLA-DQB1*0402 has been reported to be associated with the autoimmune diseases primary biliary cirrhosis, type 1 diabetes and juvenile idiopathic arthritis. Determination of PTPN22 polymorphisms did not disclose any significant

differences between NMO and MS nor controls, but did provide valuable information as PTPN22 has not been investigated previously in NMO patients. The association of a minor allele of the PD-1 gene with NMO is a novel finding, suggesting an involvement of the PD1- PD1-L pathway in the pathogenesis of NMO. The PD1-PD1-L pathway is physiologically related to T cell function (131). In summary this study presents a large set of data on HLA and the autoimmunity markers PTPN22 and PD-1, which latter markers have not been investigated previously in NMO. These data constitute important background information, stressing the autoimmune element of NMO, and should be confirmed in larger or multicenter studies.

The experimental study had some limitations. One limitation was the fact that we tested IgG only from one seropositive NMO-patient. Interestingly, this patient's NMO phenotype was largely reflected in the distribution of the lesions in the mice. This observation may be extended to IgG from different patients asking whether pathogenic changes of NMO-IgG in mice reflect the clinical status of the donor. The model should also be tested with IgG from seronegative NMO-patients, which could reveal other antibody specificities of pathogenic importance. The expected result from such experiment is that only IgG from seropositive patients will induce histopathology and disease. Lastly, clinical evaluation of the mice has been reported in pre-established EAE models which showed a worsening of the clinical features of EAE after the administration of NMO-IgG (93, 95), and in the study by Saadoun et al. (96) naïve mice showed impaired orientation after transfer of NMO-IgG corresponding to the affected cerebral hemisphere. Such evaluation of behavioral changes in the mice was not done in the present study, but will be the subject of further studies.

The strengths of the present experimental study was the demonstration that patient-derived NMO- IgG is sufficient to induce NMO-like pathology when administered by intrathecal injection directly into CSF, without concurrent immunization or surgical trauma to the CNS parenchyma. The model seems to be robust as the majority of these mice injected with NMO-IgG + huC' developed relevant lesions. The serum IgG anti-AQP4 antibodies employed in the model originated from a NMO patient who was diagnosed within the study cohort as described in Article I. IgG AQP4 antibodies were measured by an assay as described above.

AQP4 is a component of the glia limitans at the BBB and CSF - parenchymal barrier (132). If autoantibody is the disease-promoting mechanism of NMO, as supported by our results, it may be assumed that anti-AQP4 antibodies/NMO-IgG get access to CNS via the circumventricular areas, then initiate astrocyte damage consequently leading to BBB disruption. Such questions can be addressed by this NMO-model which involves a global evaluation of NMO-like lesions. Autoimmune diseases in general involve T cells both as regulatory cells and effector cells. A recent study demonstrated in rats that AQP4-specific T cells together with anti-AQP4 antibodies participated in the generation of NMO-like lesions (98). Lucchinetti and colleagues described rare CD8+ T cells in NMO lesions (42). AQP4-specific T helper cell responses may be required for the generation of IgG anti-AQP4 antibodies (133). MHC II-restricted AQP4 epitopes for CD4+ T cell recognition have been identified (134, 135), and recent data demonstrate in a Lewis rat model that AQP4-specific T cells induce brain lesions which were enhanced by co-administration of NMO-IgG (98).

In present preliminary studies a exacerbation of NMO-like lesions was observed in the spinal cord of 2D2 mice, suggesting that a pre-existing high frequency of MOG-specific T cells may synergize with AQP4 antibodies to induce extensive lesions in areas with less intense expression of AQP4 such as the spinal cord. It may be speculated that the myelin-specific T cells are involved in the demyelinating process. The specificity of involved T cells is not known, nor has it been determined whether induction of pathology in this system depends on T cells. In conclusion, specific experiments should clarify the role of autoreactive T cells in NMO pathology.

This Ph.D thesis has a translational goal as it integrates the clinical study and a subsequent experimental study aiming to understand NMO pathogenesis. Direct advantages have been the use of clinical human material (IgG and complement) in the experimental model and the knowledge of the variability of the clinical phenotype (e.g. brain lesions, particularly brainstem lesions) in the aims for the experimental model. Further usefulness of the experimental model could be a closer mechanistic understanding of the transport of IgG across the CSF-parenchymal barrier and further kinetic studies of the development of the disease process. Ultimately such experiments may improve treatment of NMO.

In conclusion, data from this Ph.D. thesis support an autoimmune background for NMO as the disease fulfills three out of four criteria for autoimmunity (autoantibodies in most patients, presence of autoantigen and transfer of disease activity by autoantibodies). However, some uncertainty still exists such as unexplained cases of seronegative NMO and the absence of convincing evidence of experimental pathogenicity of anti-AQP4 antibodies /NMO-IgG in the plasma of unprimed animals. Future studies will be needed to more clearly show the autoimmune nature of NMO both from clinical and experimental perspectives.

PERSPECTIVES

Multiple pieces of evidence has led to the recognition of NMO as a distinct disease entity within the CNS IDDs (inflammatory demyelinating disease) in the CNS including results from clinical, epidemiological, neuroimaging, genetic and immunological research. The increased understanding of the pathogenesis of NMO and characterisation of the natural course of the disease has facilitated the formulation of diagnostic criteria. These may further lead to the development of novel treatments.

Data from previous human and experimental studies suggest that anti-AQP4 antibodies/NMO-IgG are pathogenic, but do not exclude that there may be forms of NMO which either do not solely involve autoantibodies or involve antibodies with specificities other than AQP4. The presence of anti-AQP4 antibodies/NMO-IgG negative clinical NMO-cases suggest that the exact mechanisms for NMO have not been clarified. Seronegative cases of NMO could also be explained by differences in assay performance. Interest in AQP4 itself has led to more knowledge about its structure and function and to the development of new assays. Recently it has been shown that concentration-dependent binding of NMO-IgG to M1 and M23 expressed affinity variations, but with consistently higher affinity in the binding to M23, preferentially assembled in OAPs and a study reported an improvement from 70% to 97% sensitivity for NMO-IgG using an assay with M-23-expressing AQP4 transfected cells instead of M1-expressing cells (51). This study suggests that the conformational epitopes of M-23 AQP4 are the primary targets of serum anti-AQP4 antibodies/NMO-IgG. Multi-center studies comparing the different assays e.g. based on a ring test principle will be useful to delineate the clinical usefulness of these serological markers for NMO. Presently, no reference standard has been established.

Brain abnormalities as demonstrated by MRI and with clinical consequences have been reported in previous studies of NMO and also in our own descriptive findings. A closer investigation of these features could be done in a prospective study with uniform imaging techniques and with well- defined time intervals between data sampling. The association between NMO and NMO-spectrum could be investigated by establishing a clinical follow-up in a database of patients with ON, TM and isolated brainstem syndrome. It would in a prospective study be interesting to investigate all potential NMO patients despite initial brain MRIs diagnostic for MS. Such a study may also facilitate the identification of other initial symptoms including brain stem syndromes. The positive treatment effect of azathioprine in NMO patients is well established. Specific biological agents could become effective treatment strategies in NMO. Rituximab is a B cell suppressive agent which is promising, but randomized clinical trials are lacking. Multicenter treatment trials of Rituximab seem necessary due to the relative rarity of NMO. Furthermore, experimental models as our own may be well-suited to study experimental drugs (Rituximab, Natalizumab and others).

A genetic autoimmune dependency for NMO has not been clarified in detail, and larger investigations of HLA associations in NMO are missing. The associations of HLA to NMO and NMO-spectrum could provide insight into immune mechanism(s) in NMO. Furthermore, HLA gene polymorphisms in different NMO-spectrum populations could explain different clinical phenotypes. A future study could investigate if autoreactive T cells act in synergy with anti-AQP4 antibodies to exacerbate disease pathology. The presence of a frequent polymorphism of the gene for PD-1 in the NMO patients is a novel finding, suggesting the importance of the PD1-PD1-L pathway in the pathogenesis of NMO. Future studies may investigate T cell markers in cerebrospinal fluid and blood from NMO patients. Further genetic and experimental studies will be required to localize such genes and pathways relevant for NMO pathogenesis. It may be valuable to investigate whether AQP4 gene variants represent a genetic susceptibility factor for NMO and are associated with clinical variants of NMO.

Studies of experimental anti-AQP4 dependent NMO models seem attractive. The underlying molecular mechanisms of the effect of anti-AQP4 antibodies/NMO-IgG on astrocytes could be clarified in more detail. There is no convincing evidence of pathogenicity of anti-AQP4 antibodies/NMO-IgG in the blood of unprimed animals and so far all experimental models depend on a breach of BBB in the access to CNS of IgG. How do the antibodies reach the CNS in clinical NMO? Anti-AQP4 antibodies in the presence of complement lead to astrocyte injury and CNS inflammation. How do astrocytes contribute to the inflammatory response? How does inflammation develop into demyelination? Do AQP4-specific T or /and myelin-specific T cells play a role? Both T cell mediated immunity and innate immunity may be involved in NMO pathogenesis, and neither aspect of immunity has as yet been extensively investigated.

SUMMARY

Neuromyelitis optica (NMO) is an inflammatory demyelinating disease (IDD) of the central nervous system (CNS) and probably the most common non-multiple sclerosis (MS) CNS IDD. Serum immunoglobulin G autoantibodies have been identified in the majority of NMO patients with the water channel aquaporin-4 (AQP4) as their main target autoantigen. Previous studies have suggested ethnicity-based prevalence differences of NMO. The genetic background for these putative differences is not known. An HLA-association with NMO has been identified, but the association is not very pronounced. Human and experimental studies support that anti-AQP4 antibodies/NMO-IgG are involved in the pathogenesis of NMO. Previous experimental animal models have reported induction of NMO-like histopathology in animals by transfer of human anti- AQP4 antibodies/NMO-IgG.

A main goal of this PhD thesis was to perform a population-based study in a predominantly Caucasian population (in the Region of Southern Denmark) to estimate the incidence and prevalence of NMO and describe the clinical phenotypes in this population. Furthermore the aims were to investigate whether autoimmunity underlies or contributes to the pathogenesis of NMO with specific clinical, immunogenetic and experimental perspectives.

The yearly incidence rate of NMO in the population was estimated to be 0.4 per 105 person-years
(95% CI 0.30 - 0.54) and the prevalence was 4.4 per 105 (95% CI 3.1 - 5.7). The results indicated that NMO is more common in a Caucasian population than earlier believed. Clinical, radiologic and serological data were reviewed in order to establish the diagnostic accuracy of anti-AQP4 antibodies/NMO-IgG for specific syndromes in NMO. We observed assay characteristics with a sensitivity of 62% and a specificity of 100%. The diagnosis of NMO based on either the Wingerchuk 2006 criteria or the United States National Multiple Sclerosis Society 2008 criteria could be made purely on clinical grounds in a high proportion (64 %) of cases. Heterogeneity of clinical NMO manifestations including optic neuritis, longitudinal extensive transverse myelitis (LETM) and brain lesions were observed.

In the clinical immunogenetic study we observed that NMO patients had frequent co-existence of autoimmune disease and family occurrence of NMO and MS. The frequency of HLA-DQB1*0402 allele was increased in NMO and a significantly increased frequency of the PD-1.3A allele in the NMO patients were observed. The data suggest a possible genetic autoimmune dependency of NMO.

In the experimental part a novel animal model for NMO was established, utilizing the minimally invasive intrathecal route for antibody administration that does not involve blood brain barrier disruption or pre-existing CNS inflammation. Human IgG from AQP-4 antibody–positive NMO patients (from the clinical study) were injected together with human complement to study the effects on CNS. NMO-like histological lesions were observed at topographically restricted sites at the ependyma and in the parenchyma of the brainstem, cerebellum and periventricular areas. The lesions were characterized by deposition of immunoglobulin and complement, loss of AQP-4 expression and loss of reactive astrocytes co-localizing with inflammatory cell infiltration. This pattern is similar to the characteristic histological and radiologic features of human NMO lesions. Taken together this PhD combined clinical, epidemiological, neuroimaging, genetic and immunological data, which contribute to the characterization of the natural course of the disease and understanding of the pathogenesis of NMO. Practical consequences may be earlier diagnosis of NMO and better distinction of NMO from MS and other IDDs, important due to differences in prognosis and therapy.

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