**Cerebrospinal Fluid Complement System Biomarkers in Demyelinating Disease.**

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**Keywords:** Multiple sclerosis, neuromyelitis optica, cerebrospinal fluid, biomarkers, complement.

**Abbreviations:** AQP4-Ab, aquaporin-4 antibody; AUC, area under the curve; BBB, blood-brain barrier; BSA, bovine serum albumin; C1INH, C1 Inhibitor; CNS, central nervous system; CSF, cerebrospinal fluid; CIS, clinically isolated syndrome; ELISA, enzyme-linked immunosorbent assay; FB, Factor B; FH, Factor H; FHR125; FH-related proteins 1, 2 and 5; FI, Factor I; HRP, horseradish peroxidase; MOG-Ab, Myelin Oligodendrocyte Glycoprotein antibody; MS, multiple sclerosis, NMOSD, Neuromyelitis Optica Spectrum Disorder; OPD, o-Phenylenediamine dihydrochloride; PBS-T, phosphate-buffered saline-0.1% Tween20; PC1, principal component 1; PC, principal component 2; ROC, Receiver–Operating Characteristics; RT, room temperature; sPLS-DA, Sparse Partial Least Squares Discriminant Analysis; terminal complement complex TCC.

**Abstract**

Background: Multiple sclerosis (MS) can be difficult to differentiate from other demyelinating diseases, notably Neuromyelitis Optica Spectrum Disorder (NMOSD). We previously showed that NMOSD is distinguished from MS by plasma complement biomarkers.

Objective: Here we measure cerebrospinal fluid (CSF) complement proteins in MS, NMOSD, and clinically isolated syndrome (CIS), a neurological episode that may presage MS, to test whether these distinguish NMOSD from MS and CIS. Materials and methods: CSF (53 MS, 17 CIS, 11 NMOSD, 35 controls) was obtained, complement proteins (C4, C3, C5, C9, C1, C1q, Factor B (FB)) regulators (Factor I (FI), Factor H (FH), FH-Related Proteins 1, 2 and 5 (FHR125), C1 Inhibitor (C1INH), Properdin), and activation
products (terminal complement complex (TCC), iC3b) were quantified by ELISA and results expressed relative to CSF total protein (µg/mg).

Results: Compared to control CSF: I) levels of C4, C1INH and Properdin were elevated in MS; II) TCC, iC3b, FI and FHR125 were increased in CIS; III) all complement biomarkers except TCC, FHR125, Properdin and C5 were higher in NMOSD CSF. A statistical model comprising six analytes (C3, C9, FB, C1q, FI, Properdin) plus age/gender optimally differentiated MS from NMOSD.

## Introduction

Multiple sclerosis (MS) is a chronic, immune-mediated, degenerative disease of the central nervous system (CNS)**,** causing a wide range of symptoms including problems with movement, balance and vision. Other related inflammatory demyelinating diseases with overlapping features include Neuromyelitis Optica Spectrum Disorders (NMOSD) where demyelination affects predominantly the optic nerves and spinal cord resulting in loss of vision and spinal symptoms, and Clinically Isolated Syndrome (CIS), a transient first clinical episode with MS-like symptoms which may or may not be an early sign of MS. Because of the similarity of symptoms in the early phase of the three conditions, diagnosis and subsequent management can be difficult and so

there is an unmet need for biomarkers that enable early diagnosis and differentiation. Autoantibodies against the water channel aquaporin 4 (AQP4-Ab) are highly specific for NMOSD patients[1-4]; however, AQP4-Ab are absent in up to 40% of all NMOSD patients fulfilling the clinical criteria.[5] These AQP4-Ab negative NMOSD patients can present with symptoms also encountered in MS and CIS, and correct diagnosis remains challenging. We and others have shown that complement activation drives pathology in both NMOSD and MS[6-17], with evidence from immunohistochemistry[6,13-14,16,18,23] as well as studies measuring complement proteins in cerebrospinal fluid (CSF) and plasma/serum[7,8,10-12,17,19,20]. In our previous study of plasma complement biomarkers[20] we found that several complement proteins and activation products were higher in NMOSD compared to MS or controls and a model comprising C1 inhibitor (C1INH) and terminal complement complex (TCC) distinguished NMOSD from MS (area under the curve (AUC): 0.98), while C1INH and C5 distinguished NMOSD from controls (AUC: 0.94). In the current study, we have built on the published work by investigating an expanded set of complement biomarkers, including complement proteins (C4, C3, C5, C9, C1, C1q, Factor B (FB)), regulators (Factor I (FI), Factor H (FH), Factor H Related Proteins 1, 2 and 5 (FHR125), C1INH, Properdin), and activation products (TCC, iC3b) in CSF with the aim of identifying informative markers that may help to differentiate NMOSD, MS and CIS. Measurement of CSF levels of complement analytes may not only contribute to differential diagnosis in demyelination but also aid stratification and help define pathogenesis.

## MATERIALS AND METHODS

### Samples

CSF samples (n = 136) were obtained from the Welsh Neuroscience Research Tissue Bank in November 2016. Diagnosis at time of sample requisition was used to categorise as this diagnosis, which had emerged after a mean of 4.3 years of follow-up, was regarded to be more reliable that the provisional diagnosis made at the time of CSF sampling. Diagnoses made at time of sample requisition, according to contemporary diagnostic criteria, were: 53 MS, 17 CIS, 11 NMOSD and 55 non-inflammatory disease controls. Of note, because the samples were accessed in 2016, the CIS classification was based on the criteria current at that time; *post-hoc* we re-

assessed the CIS group using the 2017 criteria and only one of the 17 was differently classified (as MS) by these criteria. Relevant demographic, clinical and routine laboratory data are summarised in Table 1.

CSF red cell and white cell count, albumin and total protein were measured on freshly harvested samples and the presence of oligoclonal bands or anti-aquaporin-4 antibodies assessed where appropriate to the putative diagnosis. Samples were then centrifuged (2000g/10mins) to remove cells and debris within 30 minutes of collection before being aliquoted and frozen at –80°C until use. Serum was collected at the same time as the CSF sample, measurement of serum albumin and total protein levels were assessed where appropriate to the putative diagnosis. Ethical approval for the study was obtained from the Research Ethics Committee for Wales (Ref 09/ MRE09/35).

**Immunoassays**

Complement analytes (n = 13) were selected for this study guided by reference to previous studies of complement biomarkers in MS and availability of reagents. The concentrations of these 13 analytes: C3, C4, C5, C9, FB, FH, FI, FHR125 (measures FH-related proteins 1, 2 and 5), Properdin, C1INH, C1q, iC3b and TCC, were measured using established in-house ELISAs (Table 2). The available volumes of collected CSF varied between 0.2–0.5 ml, stored at -80°C. Nunc Maxisorp (VWR, UK) plates were coated with affinity-purified capture antibody overnight at 4°C, wells washed 1 x with phosphate-buffered saline 0.1% Tween20 (Sigma Aldrich, Germany) (PBS-T) and blocked for 1 hour at room temperature (RT) with 1% bovine serum albumin (BSA) in PBS-T. After 3 washes purified protein standards or serum samples, optimally diluted in 0.1% BSA in PBS-T, were added to wells in duplicate and incubated for 1.5 hours at 37°C. Different sample dilutions were used for different assays (Table 2). Wells were washed 3 x with PBS-T then incubated (1 hour) at RT with detection antibody (either unlabelled or labelled in-house using horseradish peroxidase (HRP; EZ-Link Plus Activated Peroxidase Kit, ThermoFisher Scientific, UK) and washed 3 x with PBS-T. For assays using unlabelled detection antibodies, HRP-labelled secondary antibody (anti-mouse or anti-rabbit IgG as appropriate, Jackson ImmunoResearch, USA) was added to wells, incubated and washed as above. Signals were detected using o-Phenylenediamine dihydrochloride (OPD;

SIGMAFAST™, Sigma-Aldrich, Germany) and absorbance (492nm) was measured. Standards were included on each plate and samples from controls and patients were randomly assigned to eliminate assay bias. A nonlinear regression model was used to fit standard curves generated by ELISA. Total protein concentration (µg/mg) was automatically calculated by reference to the standard curve using GraphPad Prism. Detection limits, working ranges and assay performance were determined as described[20] using CSF from 35 controls. In order to correct for differing degrees of blood-brain barrier impairment, all analytes were expressed relative to CSF total protein concentration (µg/mg), measured at the time of analysis using the micro-BCA Protein Assay Kit (ThermoFisher Scientific, UK).

### Statistics

Mann-Whitney tests were used to test significance of differences between the control group and each of the other three groups; MS, CIS and NMOSD. Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was used to select sets of variables that best separated the sample groups. The complement biomarkers that were the most strongly associated with diagnosis where combined into one model; Receiver–Operating Characteristics (ROC) curves were constructed for the model, and the area under the curve (AUC) for the final model was calculated and compared to that for individual biomarkers. AUC was used to define the predictive power of the biomarker set that compromised the model. Any biomarkers that were not significant in the model were excluded from the analysis. All analyses were conducted using R software, version 3.2.3, including the packages ‘dunn.test’, ‘corrplot’, ‘FactoMineR’ and ‘mixOmics’.

## RESULTS

### CSF levels of complement proteins are altered in demyelination

All complement proteins were quantified by ELISA and expressed relative to CSF total protein concentration (µg/mg) measured using the micro-BCA assay, (Table 3, Figure 1). Compared to controls: i) CSF levels of C4, C1Inh and Properdin were significantly elevated in MS CSF; ii) TCC, iC3b, FI and FHR125 were significantly increased in CSF

from CIS patients; iii) all tested complement biomarkers except TCC, Properdin, FHR125 and C5 were significantly higher in NMOSD CSF. Of note, the complement activation products iC3b and TCC were not detectable in the majority of the disease and control CSF samples (below the detection limit: Control, TCC = 97% / iC3b = 77%; MS, TCC = 89% / iC3b = 68%; NMOSD, TCC = 91% / iC3b = 27%; CIS, TCC = 76% / iC3b = 41%); these were therefore not used in the final models. It is, however, interesting to note that detectable levels of iC3b were much more frequent in CIS and NMOSD groups compared to control and MS groups. We performed subgroup analyses in MS, NMOSD and CIS. There were no significant differences in analyte levels between OCB+ and OCB- MS samples except for a higher C3 level in the OCB- subgroup; comparison of AQP4-positive and AQP4-negative NMO samples showed significantly higher levels of iC3b, C3, C5, FI and the FHR proteins in the antibody-positive samples. Patients designated CIS at the time of sample requisition were divided based on whether in the current diagnosis (24-30 months later) they had or had not converted to MS; of the analytes measured, only FI levels were significantly different between these subgroups, higher in convertors (Table 3).

**Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) discriminates between NMOSD, MS and CIS groups.**

The principal component analyses correspond to the sets of complement biomarkers that contribute most to the statistical model (Figure 2A, B). The first component (PC1) discriminates NMOSD from MS and CIS; the most contributing analytes, with the highest loadings, are C9, iC3b, C1INH and C1q. The second component (PC2) clearly discriminates between CIS and MS through two analytes; higher levels of FI (strongly positively correlated, 0.97) in the CIS group and lower levels of C5 (negatively correlated, -0.25) in MS. Most of the assays (8 out of 13) showed significant differences between the Control and NMOSD groups, with higher levels in the NMOSD group (Figure 2 A-C).

**A statistical model comprising six CSF complement analytes optimally differentiates MS from NMOSD.**

Receiver-Operating Characteristic (ROC) analyses showed no significant contribution of complement biomarkers over demographics (age + gender) for differentiating MS, NMOSD or CIS from Controls; however, complement biomarkers markedly improved differentiation of MS and NMOSD in ROC analysis (Figure 2D). The six most significant complement analytes based on p-value, C3, C9, FB, C1q, FI and Properdin, were combined in the model. The other seven analytes were removed from the model after testing as they did not significantly contribute. The model to differentiate NMOSD from MS had an AUC 0.81, considered highly predictive (Figure 2D).

## Discussion

Sensitive and specific biomarkers are essential for diagnosis, prediction and stratification of neurological diseases. Differentiating the demyelinating diseases MS and NMOSD is a particular priority because presenting symptoms can be similar but therapies that are effective in MS can exacerbate NMOSD[21]. To date, the best NMOSD biomarkers are the associated autoantibodies (AQP4-Ab and MOG-Ab); these are highly specific[22], however, a significant proportion (20 – 30%) of NMOSD cases are autoantibody-negative and these are difficult to distinguish from MS. The role of complement in pathogenesis of both MS and NMOSD is well accepted; however, its nature and magnitude remain unknown. Here we present an in-depth analysis of complement in CSF in NMOSD, MS and CIS. We measured complement components (C4, C3, C5, C9, C1, C1q, FB) regulators (FI, FH, FHR, C1INH, Properdin), and activation products (TCC, iC3b) to identify their capacity to differentiate these demyelinating diseases. Given the complex and variable immunopathogenesis of MS, NMOSD and CIS, the use of a combination of CSF analytes is much more likely to yield an informative biomarker of disease that differentiates between these three diseases than any single analyte.

CSF levels of C4, C1INH and Properdin were significantly elevated in MS compared to controls ($p < 0.05$). Increased plasma levels of complement protein C4 and classical pathway regulator C1INH in MS plasma have been demonstrated previously[12,20]. Our study supports this finding; increased levels of CSF C4 and C1INH imply ongoing complement dysregulation that is both local and systemic. CSF Properdin was also significantly increased in MS, a finding that might contribute to the perpetuation of

complement activation in the CSF through properdin stabilization of alternative pathway C3 convertases.

Although TCC and iC3b were not detectable in the majority of CSF samples with the assays used and so not further utilised in models, a surprising finding was that the proportion of CIS patients showing ongoing complement activation, evidenced by elevated levels of the activation markers iC3b and TCC, was significantly greater in comparison with controls (iC3b, 59% vs 23%; TCC, 24% vs 3%), suggesting that there was ongoing complement activation and dysregulation in the CSF in CIS. There were only 17 CIS samples in our study so this finding is preliminary; nevertheless, it does make the case for a larger study of complement activation markers in CIS to discover whether they aid diagnosis and/or help predict whether progression to MS will occur.
The three disease groups were divided into subgroups based on OCB status for MS, AQP4 antibody-positivity for NMO and conversion to MS in the CIS group. Although the numbers in the subgroups were small, some significant differences were observed. The only significant difference between OCB+ and OCB- MS subgroups was a higher C3 level in the OCB-; this might suggest increased complement consumption in the OCB+ group. AQP4+ NMO samples showed significantly higher levels of multiple complement analytes, iC3b, C3, C5, FI and the FHR proteins compared to AQP4- samples; numbers in these subgroups were small (6 and 5) so these findings are preliminary. CIS cases that subsequently converted to MS had significantly higher FI levels compared to non-convertors; again, subgroup numbers were small (5 and 12) so findings should be treated with caution. The data make the case for a similar subgroup analyses in larger sample sets.

The combination of multiple analyte measurements into a single statistical model can provide a powerful predictive test as we have previously shown for plasma markers of disease[20]. Here we have combined CSF complement biomarkers in a model to distinguish between NMOSD and MS. A model comprising a combination of six CSF complement biomarkers (C3, C4, C5, C9, FH, FHR, and C1INH) plus demographics (age and gender) was capable of distinguishing these diseases with good predictive power (ROC analysis; AUC = 0.81; Figure 2D). Partial least squares analysis (sPLS-DA) enabled the three disease groups to be distinguished (Figure 2A-C). In our previous study of plasma complement biomarkers, we showed that a statistical model

including just two plasma analytes, TCC and C1INH, differentiated NMOSD from MS with a predictive power (AUC) of 0.98[20]. We reasoned that the marked differences in plasma biomarkers between the groups were a reflection of differences in blood-brain barrier (BBB) integrity; the BBB leak in NMOSD allowing complement activation products to spill over from brain to blood. We anticipated that CSF complement biomarkers would be much more similar between the diseases, and indeed, that is what we show here. Nevertheless, the majority of measured analytes were significantly higher in NMOSD CSF compared to MS or controls, suggesting greater local complement dysregulation in NMOSD. Improved understanding of the roles of complement in demyelinating diseases may help not only in differential diagnosis but also in designing strategies for using anti-complement therapeutics to suppress local complement activation within the CNS to reduce disease severity. This approach could be particularly useful in patients with NMOSD where current therapies are of limited benefit. In line with this proposition, a complement therapeutic, the C5-blocking antibody eculizumab, was recently FDA-approved for therapy of AQP4 positive NMOSD (https://news.alexion.com/press-release/product-news/alexion-receives-fda-approval-soliris-eculizumab-treatment-adults-neuromy).

**Declaration of Conflicting Interests.** The author(s) declared the following potential conflicts of interest with respect to the research, authorship and/or publication of this article: B.P.M. has provided advice on complement to Roche and is a consultant to GlaxoSmithKline; all fees are paid to Cardiff University. N.P.R. holds research grants from Genzyme and Novartis and has received honoraria for advisory boards or support to attend educational meetings from Genzyme, Biogen, Novartis, Celgene, Merck and Roche. Other authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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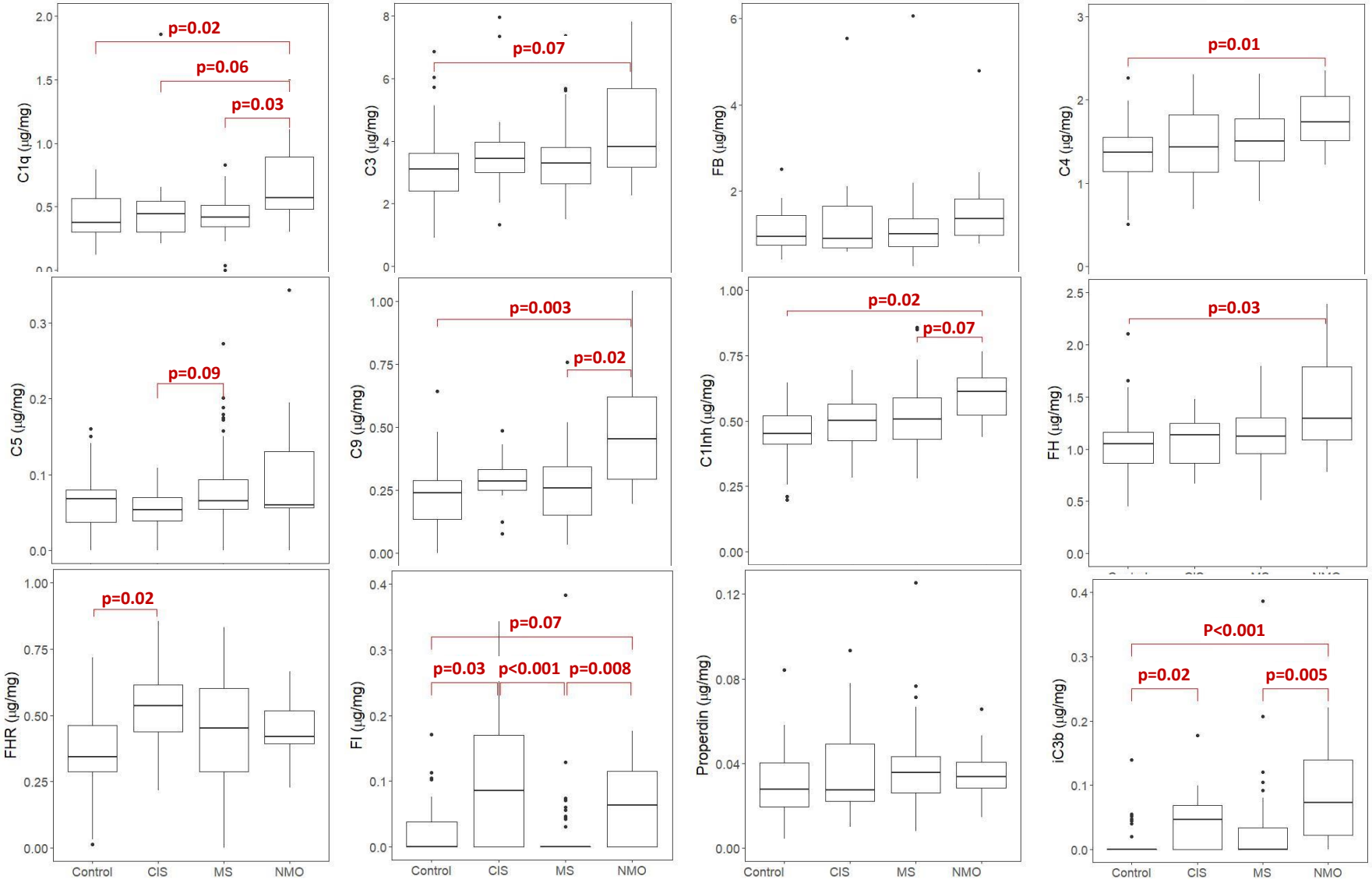
## FIGURE LEGENDS

**Figure 1: Complement protein levels in CSF samples.** Box and whisker plots are shown for each analyte except TCC where most samples were negative. CSF levels of C4, C1Inh and Properdin were significantly elevated in MS; all tested complement biomarkers except FHR, Properdin and C5 were significantly higher in NMO CSF. In CIS CSF, iC3b, FI and FHR125 were increased compared to the controls (data presented in µg/mg for all analytes). p values are presented above connecting bars for all significant inter-group differences. Abbreviations: CIS, clinically isolated syndrome; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder.

**Figure 2: sPLS-DA and ROC analyses reveal clustering of cases and discriminatory power of individual and identified sets of analytes.**
A) sPLS-DA was used to select variables that best separate the different sample groups; correlations of the variables with the Principal Components (PC1, PC2) are shown. B) PC1 best discriminates NMOSD from the other conditions while PC2 best discriminates between CIS and MS. C) The circle plot shows that NMOSD is associated with increased levels of multiple analytes (C3, C4, C5, C9, FH, FHR, and C1INH) while CIS is associated with increased FI levels. D) ROC curves comparing the model which best differentiates NMOSD from MS (Red; C3, C9, FB, C1q, FI, Properdin) with demographics (Blue; age/gender) alone. The area under the curve (AUC) for the final model, used to define predictive power for distinguishing NMOSD from MS, was 0.81. Abbreviations: AUC, area under the curve; CIS, clinically isolated syndrome; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; PC1, principal component 1; PC, principal component 2; ROC, Receiver-operating characteristic; sPLS-DA, sparse Partial Least Squares Discriminant Analysis.

**Figure 1**

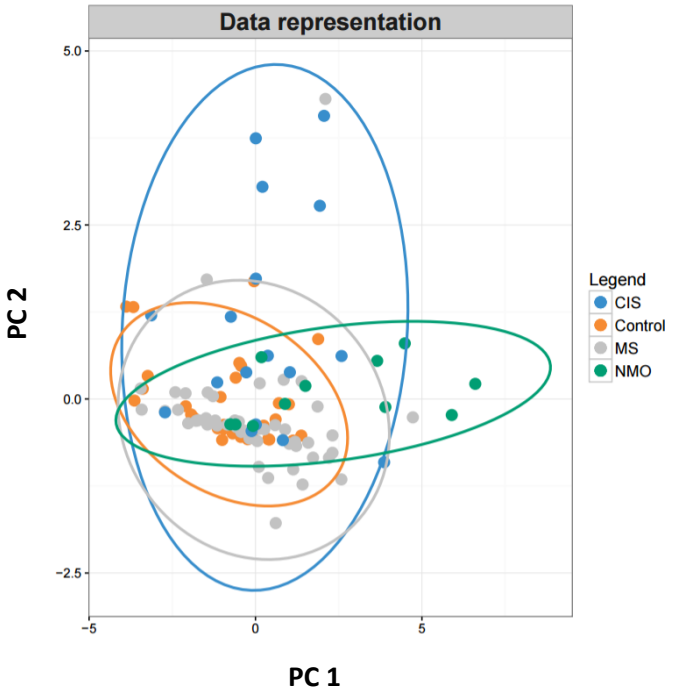

C1q (µg/mg)
p=0.02
p=0.06
p=0.03
C3 (µg/mg)
p=0.07
FB (µg/mg)
C4 (µg/mg)
p=0.01
C5 (µg/mg)
p=0.09
C9 (µg/mg)
p=0.003
p=0.02
C1Inh (µg/mg)
p=0.02
p=0.07
FH (µg/mg)
p=0.03
FHR (µg/mg)
p=0.02
FI (µg/mg)
p=0.07
p=0.03
p<0.001
p=0.008
Properdin (µg/mg)
iC3b (µg/mg)
P<0.001
p=0.02
p=0.005
Control
CIS
MS
NMO

## Figure 2

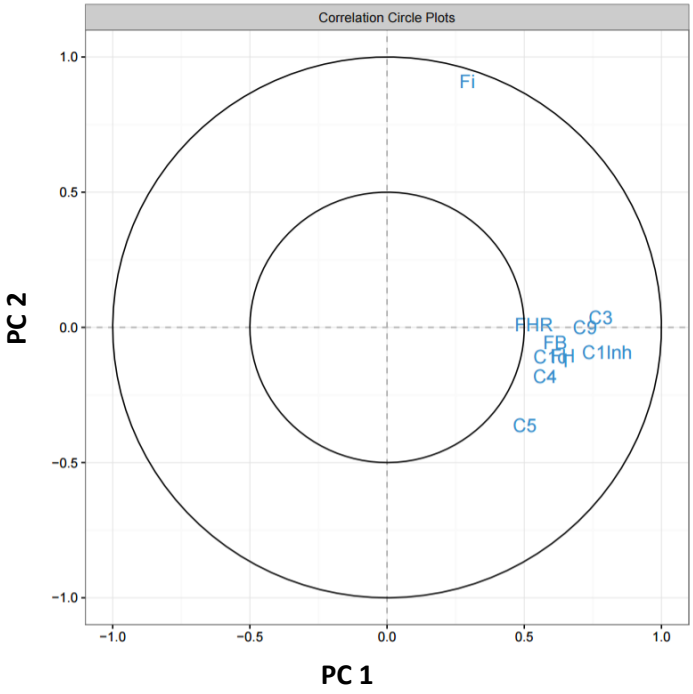
### A)

| | PC1 | PC2 |
|---|---|---|
| C4 | 0.33 | 0 |
| C3 | 0.3 | 0 |
| C1Inh | 0.38 | 0 |
| FHR | 0.19 | 0 |
| FH | 0.3 | 0 |
| FB | 0.24 | 0 |
| C1q | 0.36 | 0 |
| FI | 0.27 | 0.94 |
| Properdin | 0 | 0 |
| C9 | 0.47 | 0 |
| C5 | 0.22 | -0.35 |
| Control | -0.56 | 0.1 |
| CIS | 0.13 | 0.84 |
| MS | -0.05 | -0.53 |
| NMOSD | 0.82 | 0.04 |

### B)




### C)




### D)

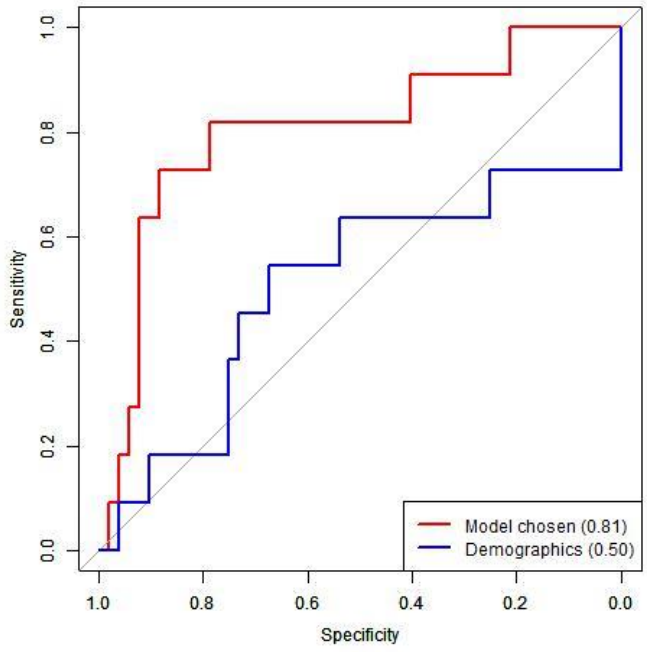


**Table 1**

| **Diagnostic category at time of analysis[v]** | **Clinically Isolated Syndrome (CIS)** | **Multiple Sclerosis (MS)** | **Neuromyelitis Optica Spectrum Disorder (NMOSD)** | **Controls** |
|---|---|---|---|---|
| **Number (n)** | 17 | 53 | 11 | 55 |
| **Age at time of sampling[vv] (mean (range) in years)** | 41.5 (17-65) | 42.9 (21-68) | 48.1 (23-78) | 35.7 (17-84) |
| **Sex (number; % female)** | 14 (82%) | 37 (70%) | 10 (91%) | 42 (75%) |
| **Disease duration at the time of sampling[vv] (mean (range) in years)** | 2.1 (0-13.2) (n=16) | 6.2 (0-45.9) (n=48) | 9.4 (0-29.7) (n=8) | n/a |
| **Disease subtype at time of sampling[vv]** | 4 Optic neuritis<br>6 Myelitis<br>7 Other | 8 CIS<br>31 RR<br>3 SP<br>10 PP | 6 AQP4 +,<br>5 AQP4 -[vvv] | 35 IIH<br>3 Non-inflammatory myelopathy<br>3 Cerebrovascular<br>2 Non-inflammatory optic neuropathy<br>8 Other<br>5 Unknown |
| **EDSS score at time of sampling[vv]** | **0.0-3.5** 14 (82%)<br>**4.0-6.0** 1 (6%)<br>**6.5-8.5** 0 (0%)<br>**NR** 2 (12%) | **0.0-3.5** 37 (71%)<br>**4.0-6.0** 9 (17%)<br>**6.5-8.5** 3 (6%)<br>**NR** 3 (6%) | **0.0-3.5** 0 (0%)<br>**4.0-6.0** 1 (9%)<br>**6.5-8.5** 6 (55%)<br>**NR** 4 (36%) | n/a |
| **Oligoclonal band +** | 2 OCB +<br>15 OCB - | 41 OCB +<br>12 OCB – | 1 OCB +<br>10 OCB - | n/a |
| **CSF total protein (mean (range) in g/L)** | 0.45 (0.21 – 1.42) (n=17) | 0.39 (0.22 – 0.78) (n=52^) | 0.59 (0.19 – 2.10) (n=10^) | 0.33 (0.17 – 0.95) (n=53^) |
| **CSF cells: RBCs**<br><br>**WBCs** | 67 (0-610)<br>4 (0-40)<br>(n=15^) | 470 (0-15736)<br>2 (0-14)<br>(n=51^) | 53 (0-330)<br>2 (0-8)<br>(n=11) | 244 (0-6398)<br>1 (0-16)<br>(n=54^) |
| **CSF/serum Albumin ratio (mean (range))** | 7.2 (2.2-26.6) (n=17) | 5.6 (2.5-13.0) (n=52) | 9.8 (2.5-35.1) (n=10) | n/a |
| **Interval from CSF sampling to biomarker** | 5.0 (1.8-9.4) | 5.4 (1.6-9.4) | 3.7 (1.9-5.8) | 3.2 (1.5-9.3) |

| | | | | |
|---|---|---|---|---|
| **analysis (mean (range) in years)** | | | | |
| **Relapse at the time of sample acquisition (number (%))** | 0 (0%) | 2 (4%) | 3 (27%) | n/a |
| **Relapse within 12 months prior to CSF sampling (number (%))** | 12 (71%) | 29 (56%) | 4 (36%) | n/a |

**Table 1:** Clinical and demographic characteristics of the cohort. **AQP4** aquaporin 4 antibody, **CIS** clinically isolated syndrome, **EDSS** Expanded Disability Status Scale, **IIH** idiopathic intracranial hypotension, **RR** relapse-remitting, **PP** primary progressive, **SP** secondary progressive, **NR** not recorded. The other diagnoses recorded in controls were: stiff person syndrome, fibromyalgia, mitochondrial myopathy, cerebellar ataxia, degenerative spinal disease, migraine, autoimmune encephalitis, normal pressure hydrocephalus. ˅At the time of sample requisition for biomarker analysis; ˅˅At the time of CSF collection; ˅˅˅Serum AQP4 antibodies were assessed at the time of CSF sampling in 5 patients and at various intervals between -26 and +23 months of CSF sampling in 6 patients. ^reduced numbers of samples for these measures because data was not recorded on some of the patients.

**Table 2**

| Assay | | Capture Antibody | Detection Antibody | Standard | Working Ranges (ng/ml) | Sample Dilution |
|---|---|---|---|---|---|---|
| 1 | **C1q** | Monoclonal anti-Human C1q *(WL02, Hycult)* | Biotinylated Monoclonal anti-Human C1q *(DJ01, Hycult)** | C1q | 32-100 | 1 in 4 |
| 2 | **C3** | Rabbit anti-C3 | Rabbit anti-C3-HRP | C3 (Comptech) | 23-1000 | 1 in 80 |
| 3 | **C4** | Goat anti-C4 | Monoclonal anti-C4-HRP | C4 (Comptech) | 8-500 | 1 in 4 |
| 4 | **C5** | Rabbit anti-C5 | Monoclonal anti-C5 (2D5)* | C5 | 32-1000 | 1 in 2 |
| 5 | **Factor B** | Monoclonal anti-FB (JC1) | Monoclonal anti-FB (MBI 5)-HRP | FB | 63-1000 | 1 in 4 |
| 6 | **Factor H** | Monoclonal anti-FH (OX24) | Monoclonal anti-FH (35H9)-HRP | FH | 16-1000 | 1 in 8 |
| 7 | **C1 Inhibitor** | Monoclonal anti Human C1 Inhibitor | Rabbit anti-C1inhibitor* | C1 Inhibitor | 4-100 | 1 in 40 |
| 8 | **Properdin** | Monoclonal anti-Properdin (1.1.1) | Monoclonal anti-Properdin (12-14-2)-HRP | Properdin (Comptech) | 7-100 | n/a (neat used) |
| 9 | **FHR125** | Monoclonal anti-FHR125 (MBL125) | Rabbit anti-FH* | FHR125 | 4-250 | 1 in 8 |
| 10 | **TCC** | Monoclonal anti-TCC *(aE11, Hycult)* | Monoclonal anti-TCC (E2)-HRP | TCC | 63-1000 | n/a (neat used) |
| 11 | **iC3b** | Monoclonal clone 9 | Monoclonal bH6 *(HM2168, Hycult)*-HRP | iC3b (Comptech) | 32-1000 | 1 in 80 |
| 12 | **C9** | Monoclonal B7 | Rabbit anti C9* | C9 | 4-100 | 1 in 8 |
| 13 | **FI** | Monoclonal 1C/11 | Monoclonal 7D4-HRP | FI (Comptech) | 63-1000 | n/a (neat used) |

**Table 2:** Conditions used in the ELISA assay kits. All antibodies and standards were produced in-house unless otherwise specified. Abbreviations: FB, Factor B; FH, Factor H; FHR125, Factor H Related Proteins 1, 2 and 5; FI, Factor I; HRP, horseradish peroxidase; TCC, terminal complement complex; Comptech, Complement Technology Inc. (https://www.complementtech.com/); Hycult, Hycult Biotech Com. (https://www.hycultbiotech.com/). ***These assays also used species-specific HRP-labelled antibody or HRP-avidin (Jackson ImmunoResearch, USA) as appropriate to label the detection antibody.***

## Table 3

| Group | Sample Number | Age (Mean) | Gender Female (%) | TCC | iC3b | FB | FH | FHR125 | FI | Properdin | C1INH | C1q | C3 | C4 | C5 | C9 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) | µg/mg (P) |
| **MS** | 53 | 43 | 72 | 0.009 (0.129) | 0.027 (0.247) | 0.880 (0.276) | 0.942 (0.196) | 0.364 (0.054) | 0.017 (0.573) | 0.033 (0.453) | 0.415 (0.027*) | 0.342 (0.264) | 2.806 (0.326) | 1.238 (0.025*) | 0.072 (0.088) | 0.213 (0.358) |
| **NMOSD** | 11 | 49 | 82 | 0.052 (0.373) | 0.085 (‹0.0001*) | 1.346 (0.013*) | 1.344 (0.019*) | 0.440 (0.054) | 0.081 (0.016*) | 0.037 (0.437) | 0.536 (0.003*) | 0.671 (0.0002*) | 4.541 (0.020*) | 1.529 (0.005*) | 0.132 (0.038*) | 0.395 (‹0.0001*) |
| **CIS** | 17 | 42 | 88 | 0.052 (0.028*) | 0.044 (0.005*) | 1.135 (0.023*) | 0.982 (0.255) | 0.506 (0.002*) | 0.121 (0.018*) | 0.039 (0.293) | 0.462 (0.038*) | 0.403 (0.031*) | 3.842 (0.061) | 1.249 (0.073) | 0.044 (0.430) | 0.273 (0.040*) |
| **Control** | 35 | 31 | 80 | 0.002 | 0.012 | 0.793 | 0.840 | 0.286 | 0.023 | 0.029 | 0.352 | 0.315 | 2.545 | 1.03 | 0.052 | 0.190 |
| **Sub-analysis** | | | | | | | | | | | | | | | | |
| **MS OCB+** | 41 | 42 | 76.2 | 0.005 | 0.024 | 1.078 | 1.156 | 0.418 | 0.084 | 0.036 | 0.495 | 0.422 | 3.244 | 1.537 | 0.075 | 0.244 |
| **MS OCB-** | 12 | 46.9 | 66.7 | 0.019 (0.052) | 0.043 (0.359) | 1.347 (0.330) | 1.133 (0.857) | 0.514 (0.161) | 0.053 (0.530) | 0.048 (0.073) | 0.566 (0.097) | 0.579 (0.148) | 4.053 (0.040*) | 1.461 (0.515) | 0.108 (0.081) | 0.328 (0.065) |
| **NMOSD+** | 6 | 57.4 | 100 | 0.010 | 0.141 | 2.131 | 1.670 | 0.531 | 0.272 | 0.042 | 0.661 | 0.010 | 5.683 | 1.691 | 0.162 | 0.602 |
| **NMOSD-** | 5 | 37.9 | 60 | 0 (n/a) | 0.024 (0.004**) | 1.111 (0.151) | 1.154 (0.080) | 0.355 (0.026*) | 0.075 (0.027*) | 0.029 (0.114) | 0.535 (0.052) | 0.666 (0.447) | 3.126 (0.010**) | 1.859 (0.488) | 0.045 (0.030*) | 0.333 (0.082) |
| **CIS Non-Converters** | 12 | 38.9 | 76.9 | 0.006 | 0.050 | 1.571 | 1.003 | 0.496 | 0.151 | 0.029 | 0.476 | 0.592 | 3.259 | 1.472 | 0.062 | 0.296 |
| **CIS Converters to MS** | 5 | 47.6 | 80 | 0.040 (0.783) | 0.030 (0.439) | 1.243 (0.793) | 1.166 (0.369) | 0.602 (0.325) | 0.444 (0.032*) | 0.046 (0.264) | 0.563 (0.085) | 0.404 (0.533) | 4.681 (0.167) | 1.320 (0.537) | 0.031 (0.078) | 0.322 (0.422) |

**Table 3:** Patient clinical characteristics and mean concentrations of complement proteins in MS, NMOSD, CIS and control CSF. In sub-analysis, NMOSD AQP4 positive (NMOSD+) vs negative (NMOSD-) and MS OCB positive (OCB+) vs negative (OCB-) and CIS non-converted vs converted to MS patients were compared. p values (in brackets) are calculated using Mann–Whitney test to compare each of the three conditions with controls or the relevant subgroups with each other in the sub-analysis; significant

differences are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$. Abbreviations: CIS, clinically isolated syndrome; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder.