Plasma Complement Biomarkers distinguish Multiple Sclerosis and Neuromyelitis Optica Spectrum Disorders

Svetlana Hakobyan[1], Sebastian Luppe[2], David R S Evans[3], Katharine Harding[2], Sam Loveless[2], Neil P. Robertson[2] and B. Paul Morgan[1]

Institutes of Infection and Immunity[1] and Neurosciences and Mental Health[2], Cardiff University School of Medicine[3], Cardiff University, Cardiff CF14 4XN, UK.

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Address correspondence to:

Professor B. Paul Morgan, Institute of Infection and Immunity, School of Medicine, Cardiff University, Heath Park, Cardiff CF144XN, UK.

Email: morganbp@cardiff.ac.uk.   Tel: (44)2920687096.

Abstract

Background: Multiple sclerosis (MS) and neuromyelitis optica spectrum disorders (NMOSD) are autoimmune demyelinating diseases distinguished clinically by selective involvement in NMOSD of optic nerves and spinal cord. Early clinical manifestations are similar, complicating clinical management. Aquaporin-4 autoantibody measurement aids diagnosis of NMOSD but is frequently negative, creating unmet need for alternative biomarkers.

Objective: We investigated whether plasma complement proteins are altered in MS and NMOSD and whether these provide biomarkers that reliably distinguish the diseases.

Methods: Plasma from 53 NMOSD, 49 MS and 69 control donors was tested in multiplex assays measuring complement activation products and proteins. Logistic regression was used to test whether combinations of complement analyte measurements distinguish NMOSD from controls and MS.

Results: All activation products were significantly elevated in NMOSD compared to either control or MS. Four complement proteins (C1inh, C1s, C5, FH) were significantly higher in NMOSD compared to MS or controls. A model comprising C1 inhibitor and TCC distinguished NMOSD from MS (area under curve (AUC) 0.98), while C1 inhibitor and C5 distinguished NMOSD from controls (AUC 0.94).

Conclusions: NMOSD is distinguished from MS by plasma complement activation. Selective complement analyte measurement enables differential diagnosis and supports the case for anti-complement therapy in NMOSD.

Introduction

Neuromyelitis optica spectrum disorder (NMOSD) describes a spectrum of rare autoimmune demyelinating disorders predominantly affecting the optic nerves, spinal cord and periependymal regions of the brain;[1] consensus diagnostic criteria were recently published.[2] Patients usually present with relapsing episodes of optic neuritis and/or longitudinally extensive transverse myelitis, or more rarely, brainstem syndromes, including intractable hiccups. The presenting features of NMOSD can be similar, and in some cases indistinguishable, from those of the much commoner CNS demyelinating disease multiple sclerosis (MS) so that misdiagnosis, particularly in early disease, is common. However, NMOSD and MS are distinct diseases, each requiring early diagnosis and specific therapeutic strategies for optimal management.[3-5]

In 2004, Lennon et al demonstrated autoantibodies against the water channel aquaporin-4 (AQP4) in the serum of patients with NMOSD.[6,7] AQP4-Abs were subsequently found to be highly specific for NMOSD and likely pathogenic.[8,9] However, a proportion of patients fulfilling clinical criteria for NMOSD, 20-40% in Caucasians and likely higher in some other ethnic groups, are AQP4-Ab negative.[10] Some of these AQP4-Ab negative patients have recently been shown to have autoantibodies against myelin oligodendrocyte glycoprotein (MOG) and demonstrate specific clinical features, which also suggest differences in pathogenesis.[11-13] It is likely that many of the residual autoantibody-negative patients harbour as yet unidentified pathological antibodies.

There is substantial evidence that complement activation drives pathology in NMOSD, particularly in AQP4-Ab positive cases. The AQP-4 autoantibody response is predominantly IgG1, a potent complement activator,[14] and complement activation products, including the

3

terminal complement complex (TCC), are deposited in NMOSD lesions.[15,16] *In vivo s*tudies

have shown that disease can be transferred to mice by infusion of patient-derived AQP4-Abs;

these cause complement-mediated astrocyte destruction followed by myelin loss, gliosis and

neuronal death.[17-19] Despite the evidence implicating complement, there is a paucity of

studies exploring either central or peripheral complement activation in patients. This is an

important knowledge gap since an understanding of complement activation in NMOSD

would not only inform understanding of pathogenesis but also provide potential biomarkers

for diagnosis and stratification for therapy. Here we have developed multiplexed assay sets

to interrogate complement activation and levels of key complement proteins in NMOSD

plasma. We demonstrate that plasma levels of complement activation products and some

components reliably differentiate NMOSD from either MS or normal controls. These findings

demonstrate that peripheral complement activation, evidenced by altered plasma markers, is

markedly greater in NMOSD than in MS; further, they show that a subset of plasma

complement biomarkers clearly differentiate the two diseases. Complement activation was

greater in autoantibody-positive NMOSD patients, but several complement analytes were

significantly different between autoantibody-negative NMOSD and MS. A model derived

from the best differentiating analytes was highly predictive with overall predictive power

(from area under the curve [AUC] in receiver-operating characteristic [ROC] analysis) of

0.94 for NMOSD versus control and 0.98 for NMOSD versus MS. Complement biomarkers

are relatively easy to measure and we suggest that an optimal set of the markers implicated

here will provide a useful additional tool for the diagnosis of NMOSD, including in the

diagnostically challenging AQP4-Ab-negative patients; critically, these measures also enable

differentiation from MS.

Methods

4

Samples: EDTA plasma samples from 40 MS patients, 54 NMOSD patients and 69 healthy controls were obtained from the Welsh Neuroscience Research Tissue Bank, Institute of Neurosciences and Mental Health, Cardiff University. Informed consent was obtained from all patients and ethical approval was gained from South East Wales Ethics Committee (ref no.05/WSE03/111). MS cases were classified as clinically definite relapsing-remitting MS on long-term follow-up according to accepted contemporary diagnostic criteria.[20] All MS cases had undergone diagnostic and follow-up magnetic resonance imaging (MRI) as part of routine clinical care. Data on age at onset, age at sampling, years of follow-up, relapse rate, expanded disability status scale (EDSS) at time of sampling, proximity of sampling to most recent relapse, and whether on disease-modifying therapy (DMT) and/or prednisolone at time of sampling were captured (**Table 1**). Five of the MS patients (3 female, two male; mean age at onset 36; follow-up 13 years) had predominantly opticospinal disease.

All patients with NMOSD were reviewed and the diagnosis confirmed by a neurologist with experience in the diagnosis and management of neuroinflammatory disorders including NMOSD (NR, SL). All NMOSD cases had undergone diagnostic and follow-up magnetic resonance imaging (MRI) as part of routine clinical care. AQP4-Ab and MOG-Ab status was assessed at the Clinical Neuroimmunology Service, John Radcliffe Hospital Trust, Oxford UK.[21,22] Thirty-nine of the NMOSD samples were AQP4-Ab positive, 7 were MOG-Ab positive and 8 were autoantibody-negative (**Table 1**).

Assay development and multiplexing. Ten complement analytes were selected for this study, 5 components or regulators (C1s, C3, C5, C1inh, FH), and 5 activation products (iC3b, C4d, Bb, C5a and TCC). Analyte choice was informed by reference to previous studies of complement biomarkers in MS, and availability of reagents; the activation marker set was chosen to interrogate classical (C4d, iC3b), alternative (Bb, iC3b) and terminal (C5a, TCC) activation pathways. For each analyte, an antibody pair was selected from commercial or in-

house sources (**Table 2**) and tested in ELISA for capacity to detect the analyte in plasma using purified proteins as standards. Selected antibody pairs were then tested in single-plex electrochemiluminescence (ECL) assays using high-bind plates from "ELISA Conversion Pack I" (MesoScale Discovery Platform [MSD], Rockville, Maryland, USA). Detection antibodies were conjugated to SULFO-TAG with ratio 1:12 according to the manufacturer's instructions. Single-plex assays were validated for reproducibility (intra- and inter-assay Coefficient of Variation [CV] <10%), sensitivity and dynamic range. For each analyte the range of plasma dilutions that enabled accurate quantitation was assessed; the optimal plasma dilution for measurement of all analytes in the set was then selected. Two five-plex plates (all analytes measured in a single well), one comprising an activation marker set and the other a component/regulator set, as noted above, were printed by MSD using the supplied capture antibodies, and re-validated for reproducibility, sensitivity and dynamic range and to confirm that all included analytes could be measured at a single plasma dilution. Each five-plex plate was also tested with mixtures of the relevant analyte standards to ensure that there was no "cross-talk" between assays.

The assay protocol was as follows: Printed plates were blocked with 150µl/well 3% BSA in PBS at 4°C overnight. Plasma samples were diluted (1: 1000 for the activation marker set; 1: 4000 for the component/regulator set) in assay buffer (PBS containing 1% BSA and 10 mM EDTA) and 25µl of diluted sample was added to duplicate wells and incubated while shaking at ambient for 60 min. After washing in PBS containing 0.01% Tween20, a mixture of the relevant SULFO-TAG-labelled detection antibodies diluted in assay buffer (1:100) was added and incubated as before. After washing, 150µl of 2x reading buffer was added to each well and ECL signal was immediately registered in a Sector S600 plate reader (MSD). A standard curve comprising six 5-fold dilutions of pre-mixed standard proteins was run in duplicate on the same plate and ECL values in plasma samples automatically converted to analyte

concentration by reference to the standard curve. A standardized plasma sample was used as inter-plate control.

Statistical methods. All statistical analysis was conducted in GraphPad Prism version 5 (La Jolla, CA, USA) and R version 3.0.2. Logistic regression was used to analyse the association of analyte concentration with diagnosis. The analytes which were most strongly associated with diagnosis (based on p value) were then combined into one model. Any analytes which were not significant after inclusion in the model were discarded. Receiver-operating characteristic (ROC) curves were drawn and area under the curve (AUC) for the final model was calculated, and compared to that for individual analytes. Clustered mixed-effects linear modelling (using the lme4 and lmerTest R packages) was used to explore the associations between analyte concentration and disease status. AUC was used to define the predictive power of individual analytes or the analyte set that comprised the model.

Results

Complement protein assays are sensitive and specific in multiplex formats. Each of the complement analyte assays translated from ELISA, through single-plex to multiplex without loss of performance. There was no detectable inter-assay interference between the different analytes in the multiplexes and intra- and inter-assay CVs were <10% for all analytes.

Complement activation products are elevated in NMOSD plasma compared to MS or healthy controls. Five complement activation products, relevant to classical (C4d, iC3b), alternative (Bb, iC3b) and terminal (C5a, TCC) pathways were measured in the available plasma samples (**Figure 1**). Three of these (Bb, iC3b, TCC) were significantly lower in MS compared with controls. Each of the measured activation products was significantly higher in NMOSD plasma compared to either MS or controls; iC3b and TCC were the strongest discriminators (each with p<0.005). Comparison of AQP4-Ab positive, MOG-Ab positive

and antibody-negative NMOSD samples showed a clear trend in values of activation markers AQP4-Ab+>MOG-Ab+>Antibody-negative for Bb, C4d and C5a and MOG-Ab +>AQP4-Ab +>Antibody-negative for iC3b and TCC (**Table 3**). Levels of each of the activation products were significantly higher in AQP4-Ab positive samples compared to MS but only TCC and iC3b were significantly different compared to MS in the MOG-Ab positive and antibody-negative NMOSD groups, likely because of the small number of samples in these groups. Five of the 40 MS patients in the cohort had opticospinal-dominant disease; there was no obvious difference in complement analyte levels in this subgroup compared to the whole MS group, although the numbers were too small for formal statistical evaluation. Clinical parameters were tested across all groups for correlation with complement analyte levels; no significant correlation with any complement analyte was seen for age at onset, follow-up years, relapse rate, proximity to relapse, or treatment at time of sampling (negative data not shown). EDSS was weakly correlated with each of the activation products in the NMOSD group; however, EDSS was high in all patients in this group, likely masking the correlation. MRI data was not available at time of sampling so was not tested as a covariate.

Complement proteins and regulators are elevated in NMOSD plasma compared to MS or healthy controls. Five complement proteins/regulators were measured in the available plasma samples (**Figure 2**). One of these, C1s, was significantly lower in MS compared to controls. Four (C1inh, C1s, C5 and FH) were significantly elevated in NMOSD plasma compared to MS and normal controls. C3 levels were lower in NMOSD plasma than in MS or normal controls. Comparison of AQP4-Ab positive, MOG-Ab positive and antibody-negative NMOSD samples showed very similar levels of increase in C1inh, C1s, C5 and FH in each of the groups (**Table 3**); C3 levels were significantly lower only in the AQP4-Ab positive NMOSD group compared to MS or controls.

A model comprising two complement analytes optimally differentiates NMOSD from

controls. Logistic regression analysis demonstrated that each of the complement analytes measured significantly distinguished NMOSD from controls. The three most significant of these based on p value, C1inh, C5 and FH, were combined in a model (**Table 4**); FH was removed from the model after testing because it did not significantly add to the model. AUC from a model to differentiate NMOSD from controls combining C1inh and C5 was 0.938, considered highly predictive (**Figure 3A**).

Two complement analytes optimally differentiate NMOSD from MS. Logistic regression analysis demonstrated that each of the complement analytes measured significantly distinguished NMOSD from MS. The three most significant of these based on p value, C1inh, FH and TCC, were combined in a model (**Table 4**); after testing, FH was removed from the model because it did not significantly add to the model. AUC from a model to differentiate NMOSD from MS combining C1inh and TCC was 0.977, considered highly predictive (**Figure 3B**).

Discussion

A plasma marker or marker set that is highly predictive of NMOSD and, critically, distinguishes NMOSD from MS, is a current unmet need. Numerous analytes, notably cytokines and other inflammatory markers, have been tested but none has yet proven reliable in replication studies (recently reviewed by Melamed et al).[23] AQP4 autoantibody tests, where available, are highly specific for NMOSD, but even the best available tests have a sensitivity no better than 80%, and even lower in some ethnic groups, because of the frequency of AQP4-Ab negative patients.[22,23] Some of these are positive for MOG autoantibodies (7/54 in our series);[21,24] however, a significant proportion (8/54 in our series) remain autoantibody negative, although it is likely that these harbour as-yet unidentified pathological autoantibodies. Critically, AQP4 autoantibody titres correlate poorly with

9

disease severity, response to therapy or outcome.[25] We reasoned that complement represented a likely source of biomarkers given the evidence, summarised in the introduction, that complement drives pathology in NMOSD, and the recent demonstration that anti-complement therapies impact the disease.[26,27] We explored whether selected complement analytes, or a composite signature derived from complement biomarkers, might provide a test that could supplement autoantibody measures in order to improve diagnostic certainty, particularly in AQP4-Ab negative NMOSD, and enable reliable differentiation from MS thereby reducing the possibility of iatrogenic therapeutic morbidity.[4,5,28]

A limited number of studies have examined complement proteins and activation products in NMOSD with conflicting results. In 2011, Tuzun et al measured Bb, C4d and TCC in plasma from 28 NMOSD and 29 MS cases;[29] all three of these activation markers were elevated in NMOSD compared to MS or healthy controls, the latter two significantly, leading the authors to suggest that NMOSD differed from MS "by predominance of complement system involvement". Kuroda et al measured C3a, C4a and C5a in serum and CSF from NMOSD in relapse (15), MS in relapse (15) and other neurological disease (OND) controls (12);[30] none of the serum markers were significantly different between the groups but CSF C5a was significantly elevated in NMOSD compared to MS and OND and correlated with lesion number detected by imaging. Wang et al also reported increased CSF levels of C5a and TCC in NMOSD.[31] Veszeli et al described a "systematic analysis" of complement in 25 AQP4-Ab positive NMOSD patients in remission compared to healthy controls;[32] classical and lectin pathway activities were higher and complement proteins C3, FI, FB and properdin were reduced in NMOSD plasma. Surprisingly, activation products C3a, C3bBbP and TCC were reduced in NMOSD plasma compared to controls. C1inh and C5 were measured in this study and were not different in NMOSD compared to controls. Nytrova et al measured C3a, C4a,

10

TCC and anti-C1q in anti-Aqp4-positive NMOSD (19), MS (35) and control (40) plasmas;[33] C3a and TCC were significantly increased in NMOSD compared to controls but not MS while C4a was significantly reduced in NMOSD compared to both MS and controls. C3a correlated with disease severity in NMOSD and differentiated patients with recent (past 6 months) relapse from those without recent relapse. Together, these publications reveal a lack of consensus regarding the relevance of complement and complement activation in NMOSD; although the majority describe elevated levels of complement activation products centrally in CSF and/or peripherally in plasma, some describe the opposite, provoking concern about the reliability of assays.

Using well-validated and quality-controlled multiplex assays, we found clear evidence of peripheral complement activation in NMOSD; each of the activation markers, indicative of classical (C4d, iC3b), alternative (Bb, iC3b) and terminal (C5a, TCC) pathway activation, was significantly elevated in NMOSD plasma compared to either MS or control plasma. Notably, none of the activation pathway markers was significantly elevated in MS plasma compared to controls. We previously reported that plasma levels of Bb and TCC were not elevated in MS compared to controls, while C4a was elevated but only in acute relapse.[34,35] These data demonstrate that complement activation represents a fundamental difference between the two diseases; peripheral evidence of complement activation (impacting all pathways) is specific to NMOSD. We concur with the conclusion of Tuzun et al that NMOSD differs from MS "by predominance of complement system involvement".[29] In our study, levels of activation products were highest in autoantibody-positive disease, either AQP4-Ab or MOG-Ab; each of the activation products was increased in AQP4-Ab positive NMOSD compared to MS but only TCC and iC3b were significantly increased in the MOG-Ab positive and antibody-negative NMOSD groups compared to MS. The demonstration of

11

ongoing complement activation in the antibody-negative group supports the suggestion that these individuals harbour as yet unidentified pathogenic autoantibodies. These complement activation markers measured individually provide strong differentiation between the diseases. The complement proteins C1inh, C1s, C5 and FH were significantly elevated in NMOSD plasma regardless of autoantibody status compared to MS and normal controls, while C3 levels were lower only in AQP4-Ab positive NMOSD. The mechanism(s) underpinning these changes are uncertain but likely represent the balance between increased synthesis as a part of the acute phase response and increased consumption due to complement activation.

In order to identify the most predictive set of complement analytes from our data, we generated models that selected combinations of analytes that provided complementary information to increase discriminatory power. The first model compared NMOSD cases with MS cases and included the three most significant analytes C1inh, FH and TCC; FH did not improve the model and was discarded. ROC curves constructed using just C1inh and TCC gave an AUC of 0.977 for distinguishing NMOSD from MS, considered "highly predictive".[36] The second model compared NMOSD cases with controls and included the three most significant analytes FH, C1inh and C5; FH did not improve the model and was discarded. ROC curves constructed using just C1inh and C5 gave an AUC of 0.938 for distinguishing NMOSD from controls, considered "highly predictive". These findings demonstrate that measurement of just three complement analytes in patient samples, a simple task with modern multiplexing, provides a near-perfect predictive test for diagnosis of NMOSD and for distinguishing NMOSD from MS. The findings suggest that measurement of complement analytes together with autoantibodies may in the future become accepted clinical practice in the diagnosis and monitoring of NMOSD; however, further work is needed to replicate these findings in independent cohorts and to establish whether

complement analytes are associated with disease activity, suggested here for the activation products.

We have here shown that combinations of complement biomarkers can aid diagnosis of demyelinating diseases and enable differentiation between MS and NMOSD. Importantly, complement activation and protein biomarkers were elevated not only in autoantibody-positive NMOSD but also in autoantibody-negative cases, albeit to a lesser degree, providing potential biomarkers for this difficult patient subset and revealing clues to the underlying pathology. These findings suggest that complement activation occurs even in the absence of detectable autoantibodies, a finding that may impact understanding of pathogenesis in autoantibody-negative NMOSD. These data may also influence the future use of complement inhibitory drugs in NMOSD where studies to date have been restricted to AQP4-Ab positive disease.[26]

Conflict of interest: The authors declare that they have no conflicts of interest.

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Feature	NMOSD						MS		Healthy Control	
	With AQP4-Ab		Without AQP4-Ab							
			With MOG-Ab		Seronegative					
Number	39		6		8		40		69	
Female, n (%)	37	(94.9)	5	(83.3)	3	(27.3)	31	(63.3)	44	(63.8)
Caucasian, n (%)	36	(92.3)	6	(100)	6	(100)	35	(87.5)	65*	(94.2)
Age at sampling, mean (SD)	53.8	(17.2)	43.5	(18.4)	53.0	(11.3)	41.2	(9.2)	35.6	(8.2)
Age at onset, mean (SD)	41.7	(17.9)	31.4	(14.6)	41.4	(17.4)	31.6	(10.2)	NA	NA
Follow up, mean (SD)	10.5	(8.5)	10.5	(8.9)	12.0	(15.0)	13.2	(2.8)	NA	NA
Attacks per patient, mean (SD)	6.0	(4.8)	6.1	(5.0)	5.1	(2.9)	8.2	(6.3)	NA	NA
Annualised relapse rate, mean (SD)	0.79	(0.5)	0.81	(0.5)	1.05	(0.8)	0.62	(0.5)	NA	NA

Table 1: Demographic and clinical profile of patients and controls enrolled in the study.

Abbreviations: AQP4-Ab, seropositive for IgG against aquaporin-4; MOG-Ab, seropositive for IgG against myelin-oligodendrocyte glycoprotein; NA, Not Applicable; MS, multiple sclerosis; NMOSD, Neuromyelitis Optica Spectrum Disorder; SD, standard deviation. * The ethnicity of 4 of the healthy controls was not recorded.

Assay	Capture antibody	Detection antibody	Standard	Working range (ng/ml)	Sample dilution
Activation marker set					
Bb	MM anti-neo Bb (Quidel)	MM anti-FB (JC1, in-house)	Bb Fragment (Comptech)	1.6-5000	1 in 1000
C4d	MM anti-neo C4d (Quidel)	MM anti-C4d (Quidel)	Recombinant C4d (Gifted)*	1.6-5000	1 in 1000
iC3b	MM anti-neo iC3b (Hycult)	MM anti-C3 (C3-30, in-house)	iC3b (Comptech)	1.6-5000	1 in 1000
C5a	MM anti-neo C5a (Hycult)	MM anti-C5 (Hycult)	C5a desArg (Comptech)	1.6-5000	1 in 1000
TCC	MM anti-neo C9 (aE11, Hycult)	MM anti-C8 (E2, in-house)	TCC (in-house purified)	1.6-5000	1 in 1000
Component set					
C1-INH	MM anti-C1inh (KK, in-house)	MM anti-C1inh (3C3, in-house)	C1inhibitor (Cinryze drug)	0.32-1000	1 in 4000
C1s	MM anti-C1s (M81, Hycult)	MM anti-C1s (F33, in-house)	C1s proenzyme (Comptech)	0.32-1000	1 in 4000
C3	MM anti-C3 (HC3-1, in-house)	RP C3 (in house)	C3 (Comptech)	0.32-1000	1 in 4000
C5	RP anti-human C5 (in-house)	MM anti-C5 (Hycult)	C5 (Comptech)	0.32-1000	1 in 4000
FH	MM anti-FH (OX-24, in-house)	RP anti-FH (in-house)	FH (in-house purified)	0.32-1000	1 in 4000

Table 2: Design of assays used in the multiplex sets.

The table lists the antibody pairs used in the multiplex sets, the sources of the antibodies and the standards, dilutions and assay working range. * A kind gift from Dr D Isenman, Toronto, Canada. Quidel, https://www.quidel.com/; Hycult, http://www.hycultbiotech.com/; Comptech, http://www.complementtech.com/. Abbreviations: MM, mouse monoclonal antibody; RP, rabbit affinity purified polyclonal antibody.

Analyte	Anti-Aqp-4 +ve (n=39) Mean ± SD	Anti-MOG +ve (n=6) Mean ± SD	Autoantibody -ve (n=8) Mean ± SD
Activation marker set			
Bb	3.37 ± 2.19	2.94 ± 1.93	2.22 ± 1.11
C4d	3.46 ± 1.80	2.94 ± 1.70	2.38 ± 0.71
C5a	0.03 ± 0.02	0.02 ± 0.01	0.01 ± 0.00
TCC	0.72 ± 0.33	0.86 ± 0.36	0.66 ± 0.37
iC3b	77.60 ± 25.54	81.12 ± 33.29	75.46 ± 38.64
Component set			
C1-INH	169.90 ± 26.25	182.20 ± 33.17	169.00 ± 38.40
C1s	136.20 ± 37.51	127.70 ± 31.48	98.59 ± 31.67
C3	706.30 ± 176.40	780.10 ± 219.90	864.60 ± 246.60
C5	165.00 ± 31.08	163.70 ± 32.99	117.50 ± 39.66
FH	322.20 ± 75.38	339.10 ± 45.83	268.40 ± 87.87

Table 3. Complement activation products and components in NMOSD subgroups. All

values are mean concentration in µg/ml plus or minus one standard deviation (SD).

	β (95% CI)	P
MS NMOSD versus controls		
C1inh	0.03 (0.01-0.06)	0.00352
C5	0.04472 (0.02-0.07)	9.75×10^{-5}
NMOSD versus		
C1inh	0.06 (0.02 - 0.09)	0.001278
TCC	9.60 (5.51 - 13.68)	0.000622

Table 4. Regression analysis NMO versus controls and NMOSD versus MS. Clustered

mixed-effects linear modelling (using the lme4 and lmerTest R packages) was used to explore

the associations between analyte concentration and disease status. The analytes which were

most strongly associated with diagnosis (based on p value) were then combined into the final

model. Any analytes which were not significant after inclusion in the model were discarded.

Figure 1. Plasma levels of activation products in NMOSD and MS patients compared with normal controls. Plasma concentrations of classical (C4d, iC3b), alternative (Bb, iC3b) and terminal (C5a, TCC) pathway activation products were measured in NMOSD (53 samples), MS (40 samples) and controls (69 samples). Results are in μg/ml for all analytes. Significant differences between groups are shown; *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001. The inserted Table summarises means and SDs for each analyte.

Figure 2. Plasma levels of complement components and regulators in NMOSD and MS patients compared with normal controls. Plasma concentrations of selected complement proteins and regulators were measured in NMOSD (53 samples), MS (40 samples) and controls (69 samples). Results are in μg/ml for all analytes. Significant differences between groups are shown; *, p<0.05; **, p<0.01; ***, p<0.005; ****, p<0.001. The inserted Table summarises means and SDs for each analyte.

Figure 3. Receiver-operating characteristic (ROC) curves representing models which differentiates NMOSD from controls (A) and NMOSD from MS (B). ROC curves were drawn for the final models distinguishing NMOSD from controls (A; C1inh and C5) and NMOSD from MS (B; C1inh and TCC). The area under the curve (AUC) for the final model was calculated, and compared to that for the individual analytes. AUC was used to define the predictive power of the analyte or analyte set that comprised the model; the predictive power of the model for distinguishing NMOSD from MS was 0.977, and for distinguishing NMOSD from controls was 0.938.
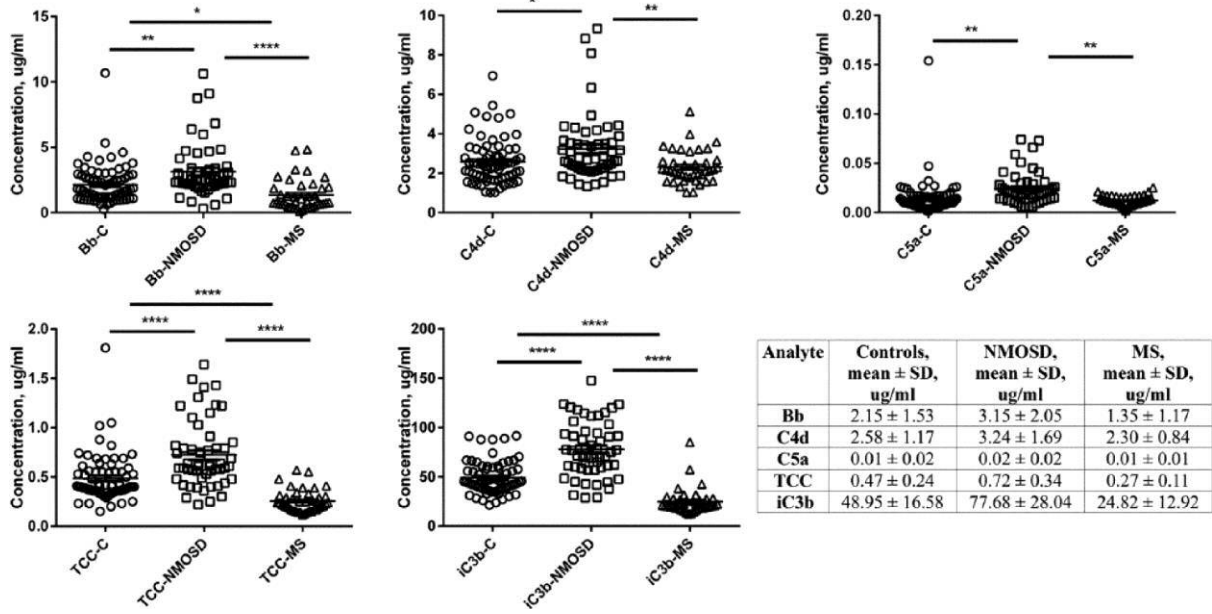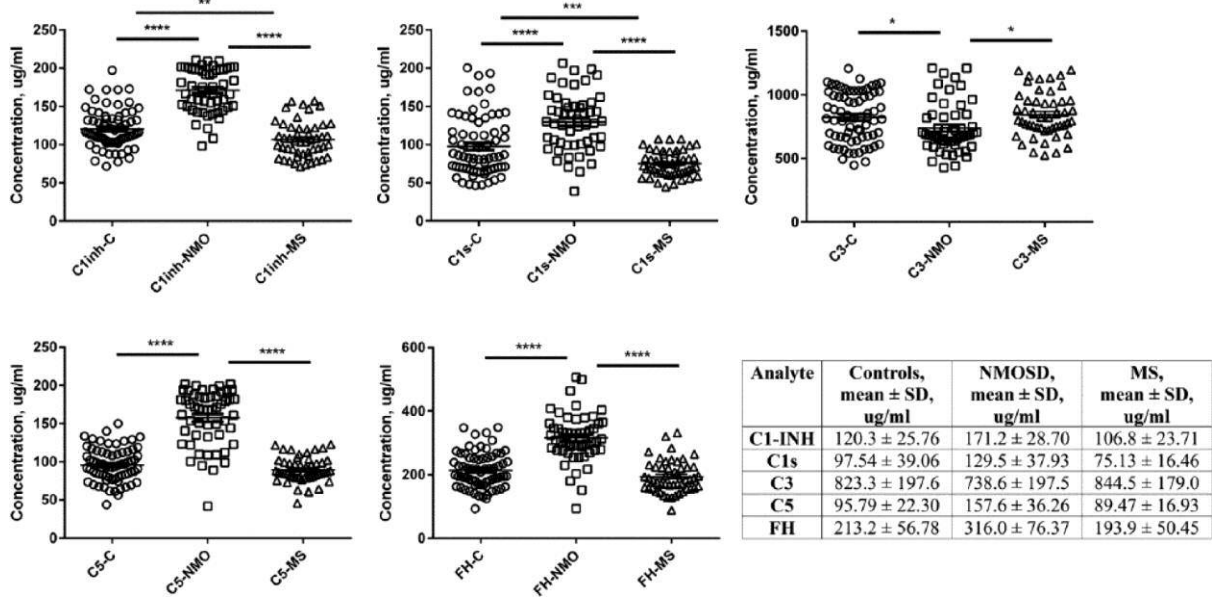
Figure 1

Analyte	Controls, mean ± SD, ug/ml	NMOSD, mean ± SD, ug/ml	MS, mean ± SD, ug/ml
Bb	2.15 ± 1.53	3.15 ± 2.05	1.35 ± 1.17
C4d	2.58 ± 1.17	3.24 ± 1.69	2.30 ± 0.84
C5a	0.01 ± 0.02	0.02 ± 0.02	0.01 ± 0.01
TCC	0.47 ± 0.24	0.72 ± 0.34	0.27 ± 0.11
iC3b	48.95 ± 16.58	77.68 ± 28.04	24.82 ± 12.92

Figure 2

Analyte	Controls, mean ± SD, ug/ml	NMOSD, mean ± SD, ug/ml	MS, mean ± SD, ug/ml
C1-INH	120.3 ± 25.76	171.2 ± 28.70	106.8 ± 23.71
C1s	97.54 ± 39.06	129.5 ± 37.93	75.13 ± 16.46
C3	823.3 ± 197.6	738.6 ± 197.5	844.5 ± 179.0
C5	95.79 ± 22.30	157.6 ± 36.26	89.47 ± 16.93
FH	213.2 ± 56.78	316.0 ± 76.37	193.9 ± 50.45

22

Figure 3

(a) NMO versus controls

(b) NMO versus MS