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► **To cite this version:**

S. Carta, A. Dinoto, M. Capobianco, P. Valentino, F. Montarolo, et al.. Serum Biomarker Profiles Discriminate AQP4 Seropositive and Double Seronegative Neuromyelitis Optica Spectrum Disorder.. Neurology Neuroimmunology & Neuroinflammation, 2023, Neurology(R) Neuroimmunology & Neuroinflammation, 11, pp.e200188. 10.1212/NXI.0000000000200188 . hal-04633004

HAL Id: hal-04633004

<https://hal.univ-lille.fr/hal-04633004>

Submitted on 5 Jul 2024

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Serum Biomarker Profiles Discriminate AQP4 Seropositive and Double Seronegative Neuromyelitis Optica Spectrum Disorder

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Neurol Neuroimmunol Neuroinflamm 2024;11:e200188. doi:10.1212/NXI.000000000200188

Abstract

Background and Objectives

Glial fibrillary acidic protein (GFAP) and neurofilament light chain (NfL) serum levels are useful to define disease activity in different neurologic conditions. These biomarkers are increased in patients with aquaporin-4 antibody–positive NMOSD (AQP4+NMOSD) during clinical attacks suggesting a concomitant axonal and glial damage. However, there are contradictory results in double seronegative NMOSD (DS-NMOSD). The aim of this study was to characterize the neuronal, axonal, and glial damage of DS-NMOSD in comparison with AQP4+NMOSD.

Methods

Patients with DS-NMOSD (i.e., for AQP4 and myelin oligodendrocyte glycoprotein antibodies—MOG-Abs) and age-matched AQP4+NMOSD diagnosed according to the latest diagnostic criteria and with available serum samples obtained within 3 months from onset/relapse were retrospectively enrolled from 14 international centers. Clinical and radiologic data were collected. Serum NfL, GFAP, tau, and UCH-L1 levels were determined using an ultra-sensitive paramagnetic bead–based ELISA (SIMOA). Statistical analysis was performed using nonparametric tests and receiver-operating characteristic (ROC) curve analysis.

Results

We included 25 patients with AQP4+NMOSD and 26 with DS-NMOSD. The median age at disease onset ($p = 0.611$) and female sex predominance ($p = 0.072$) were similar in the 2 groups. The most common syndromes at sampling in both AQP4+NMOSD and DS-NMOSD were myelitis (56% vs 38.5%) and optic neuritis (34.6% vs 32%), with no statistical differences ($p = 0.716$). Median EDSS at sampling was 3.2 (interquartile range [IQR] 2–7.7) in the AQP4+NMOSD group and 4 (IQR [3–6]) in the DS-NMOSD group ($p = 0.974$). Serum GFAP, tau,

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Go to [Neurology.org/NN](https://www.neurology.org/NN) for full disclosures. Funding information is provided at the end of the article.

The Article Processing Charge was funded by the authors.

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Glossary

AQP4 = aquaporin-4; **CBA** = cell-based assay; **GFAP** = glial fibrillary acidic protein; **IQR** = interquartile range; **MOG** = myelin oligodendrocyte glycoprotein; **NfL** = neurofilament light chain; **ROC** = receiver-operating characteristic.

and UCH-L1 levels were higher in patients with AQP4+NMOSD compared with those with DS-NMOSD (median 308.3 vs 103.4 pg/mL $p = 0.001$; median 1.2 vs 0.5 pg/mL, $p = 0.001$; and median 61.4 vs 35 pg/mL, $p = 0.006$, respectively). The ROC curve analysis showed that GFAP, tau, and UCH-L1, but not NfL, values were able to discriminate between AQP4+ and DS-NMOSD (area under the curve (AUC) tau: 0.782, $p = 0.001$, AUC GFAP: 0.762, $p = 0.001$, AUC UCH-L1: 0.723, $p = 0.006$). NfL levels were associated with EDSS at nadir only in patients with AQP4+NMOSD.

Discussion

Serum GFAP, tau, and UCH-L1 levels discriminate between AQP4+NMOSD and DS-NMOSD. The different biomarker profile of AQP4+NMOSD vs DS-NMOSD suggests heterogeneity of diseases within the latter category and provides useful data to improve our understanding of this disease.

Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is an inflammatory disease usually characterized by recurrent episodes of severe optic neuritis and/or transvers myelitis. Most patients have pathogenetic antibodies that target a water channel expressed on the end-feet surface of astrocytes (named aquaporin-4 antibodies [AQP4-Abs]), leading to astrocyte damage and subsequent demyelination and neuronal loss.¹ Recently, among AQP4-Abs seronegative cases, a subgroup of patients with a suggestive clinical phenotype was found to harbor antibodies directed against myelin oligodendrocyte glycoprotein (MOG-Abs). These antibodies define a different inflammatory disease (MOG antibody-associated disease, MOGAD), which, despite some overlapping clinical characteristics, has a different pathogenesis and disease course.^{2,3} A small subgroup of the remaining patients fulfill the latest diagnostic criteria for seronegative NMOSD and lack AQP4-Abs or MOG-Abs, which we term double seronegative NMOSD (DS-NMOSD).¹

Recently, serum biomarkers of glial and axonal damage have been investigated as possible markers of disease activity in different neurologic conditions, including AQP4-Abs-positive NMOSD (AQP4+NMOSD). The most studied molecules are glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed by astrocytes,⁴ and neurofilament light chain (NfL), involved in the structural stability and radial growth of axons.⁵ GFAP and NfL are released in the CSF after astroglial and neuronal damage, respectively. A small proportion of these proteins cross the blood-brain barrier and can be detected in serum using ultrasensitive assays, such as single molecule arrays (Simoa).^{6,7} Serum GFAP and NfL levels are higher in patients with AQP4+NMOSD compared with healthy controls, with GFAP being a reliable biomarker of disease activity.⁸⁻¹¹ Tau proteins are microtubule-associated molecules involved in the structural stability of axons and oligodendrocytes.¹² Few studies investigated its role as a

potential biomarker and reported increased tau values in MOGAD during relapses in correlation with disability.¹³ Ubiquitin C-terminal hydrolase L1 (UCH-L1) is a deubiquitinating enzyme that plays an important role in the ubiquitin-proteasome pathway.¹⁴ Its role as a potential biomarker in NMOSD has not been investigated, yet.

At present, only few studies with small sample size have analyzed the biomarker profile of seronegative NMOSD, with conflicting results.^{10,15}

The aim of this study was to characterize the neuronal, axonal, and glial damage of DS-NMOSD in comparison with AQP4+NMOSD to define the biomarker profile and pathophysiology of this condition.

Methods

Study Design

GFAP, NfL, tau, and UCH-L1 levels were blindly analyzed on stored serum samples of patients with AQP4+NMOSD and DS-NMOSD collected during an acute event by included centers and then referred to the Neuroimmunology Laboratory, University of Verona.

Patients and Samples

Adults (aged 18 years and older) with DS-NMOSD and age-matched AQP4+NMOSD fulfilling the most recent diagnostic criteria¹ and with available serum samples obtained within 3 months from an acute event (i.e., onset/relapse), defined as index event, were retrospectively enrolled from 14 centers (France, the United States, Spain, and Italy). An index event was defined as the occurrence of new symptoms or exacerbation of existing symptoms persisting for at least 24 hours and confirmed by neuroimaging and/or visual evoked potential in the absence of fever and/or infection. Samples were collected over a period of 20 years and stored at -80°C until the assays were performed. If multiple attacks occurred

and multiple serum samples were available, only serum collected during the first attack was considered. An extensive workup including infectious, rheumatologic, metabolic/genetic, vascular, and neoplastic screening was performed in patients with DS-NMOSD to rule out alternative diagnoses according to the clinical presentation. The fulfillment of the 2015 NMOSD diagnostic criteria¹ was revised centrally by 2 expert neurologists in each patient with DS-NMOSD included.

AQP4-IgG Testing

Serum samples were tested for AQP4-Abs through a live cell-based assay (CBA) quantified by either flow cytometry or microscope immunofluorescence in the reference laboratory of each recruiting center.¹⁶⁻¹⁸ DS-NMOSD samples with elevated GFAP levels were blindly retested using the same stored sera with AQP4 live CBA in a different laboratory (Innsbruck, Austria) to confirm the serostatus.

MOG-Abs Testing

Serum samples were tested for MOG-Abs through live CBA quantified by either flow cytometry or microscope visual score evaluation in immunofluorescence in the reference laboratory of each recruiting center.¹⁷⁻¹⁹

Serum NfL, GFAP, Tau, and UCH-L1 Levels

NfL, GFAP, tau, and UCH-L1 values were determined in serum samples stored at -80°C by investigators blinded to clinical data using the SR-X immunoassay analyzer (Quantex, Simoa, Lexington, MA), which runs ultrasensitive paramagnetic bead-based enzyme-linked immunosorbent assays. Analysis was performed at the Neuropathology and Neuroimmunology Laboratory, University of Verona, Italy, according to manufacturer's instructions.

Clinical Data

Clinical and paraclinical information were collected in a dedicated database by referring physicians. Data included (1) demographic information (sex, age at onset, and age at sampling); (2) clinical information on previous events (phenotype at onset, number of relapses before sampling, and last EDSS before sampling); (3) clinical information at the index event (clinical phenotype; visual acuity collected through the Snellen chart, in case of bilateral optic neuritis the worst eye was considered; EDSS at the nadir of attack; acute treatment of the index attack; and administration of chronic treatment before the index event); (4) paraclinical information (CSF cell count and protein concentration and number of vertebral segments involved in cases presenting with myelitis at the index event); and (5) follow-up information (duration of follow-up, occurrence of relapses, ongoing chronic treatment at last evaluation, and EDSS at last follow-up).

Statistical Analysis

Descriptive statistics were performed using median (interquartile ranges [IQR]) and percentages for categorical variables. Group comparisons (AQP4+NMOSD and DS-NMOSD) were assessed using nonparametric tests (χ^2 and Mann-Whitney tests), as appropriate. Correlation analyses between biomarkers and relevant clinical features were performed using 2-tailed Spearman analysis with a Bonferroni correction for multiple comparisons. Receiver-operating characteristic (ROC) curve analysis was performed to verify the discriminative power of each biomarker in differentiating AQP4+NMOSD and DS-NMOSD. Analyses were performed using IBM SPSS 25; *p* values < 0.05 were considered statistically significant.

Table 1 Demographic and Clinical Data at Onset in the Analyzed Cohort

	Whole cohort (n = 51)	AQP4+NMOSD (n = 25)	DS-NMOSD (n = 26)	<i>p</i> Value
Age at disease onset, median [IQR]	36.2 [27.0-49]	35.1 [27.4-48]	39 [24.1-50.5]	0.611
Female, n (%)	37 (72.5)	21 (84)	16 (61.5)	0.072
Clinical phenotype at onset, n (%)				
Unilateral optic neuritis	15 (29.4)	6 (24)	9 (34.6)	0.344
Bilateral optic neuritis	3 (5.9)	3 (12)	0 (0)	
Myelitis	23 (45.1)	13 (52)	10 (38.5)	
Area postrema syndrome	1 (2)	0	1 (3.8)	
Acute brainstem syndrome	1 (2)	1 (4)	0	
Focal cerebral syndrome	1 (2)	0	1 (3.8)	
Optic neuritis + myelitis	4 (7.7)	1 (4)	3 (11.5)	
Myelitis + brainstem syndrome	2 (3.9)	1 (4)	1 (3.8)	
Bilateral optic neuritis + cerebral syndrome	1 (2)	0	1 (3.8)	

Abbreviations: AQP4 = aquaporin 4; DS = double seronegative; IQR = interquartile range; NMOSD = neuromyelitis optica spectrum disorder.

Table 2 Comparison of Clinical Data and Biomarker Profiles at the Index Event Between AQP4+NMOSD and Seronegative NMOSD

	Whole cohort (n = 51)	AQP4+NMOSD (n = 25)	DS-NMOSD (n = 26)	p Value
Age at sampling median, [IQR]	40.9 [29.2–50.9]	36.8 [29.4–48.6]	42.8 [27.6–53.4]	0.624
Disease duration at sampling, mo, median, [IQR]	27.1 [3.1–99]	37.1 [2.4–103.6]	23 [5.0–99.2]	0.910
Time from previous attack to index event, mo, median, [IQR]	13 [5–37]	7.8 [4.5–51.5]	16 [5.4–42]	0.757
Sample obtained at onset, n (%)	14 (28.4)	8 (32)	6 (24)	0.529
Time from symptom onset to sampling, d, median, [IQR]	23 [9–66]	40 [10–66]	16.5 [8.3–60.8]	0.465
Chronic treatment before sampling, n (%)	22 (43.1)	13 (52)	8 (32)	0.152
Number of attacks before sampling, median, [IQR]	1 [0–3]	2 [0–4]	1 [0.8–3]	0.532
Last EDSS before the index event, median, [IQR]	3 [2–6]	2.3 [1–6]	3 [2–6]	0.646
Phenotype at sampling, n (%)				
Unilateral optic neuritis	15 (29.4)	7 (28)	8 (30.8)	0.716
Bilateral optic neuritis	2 (3.9)	1 (4)	1 (3.8)	
Myelitis	24 (47.1)	14 (56)	10 (38.5)	
Acute brainstem syndrome	1 (2)	0	1 (3.8)	
Focal cerebral syndrome	1 (2)	0	1 (3.8)	
Optic neuritis + myelitis	3 (5.9)	1 (4)	2 (7.7)	
Myelitis + brainstem syndrome	3 (5.9)	2 (8)	1 (3.8)	
Bilateral optic neuritis + cerebral syndrome	1 (2)	0	1 (3.8)	
Other	1 (2)	0	1 (3.8)	
EDSS at index event, median, [IQR]	3.5 [2.5–7]	3.2 [2–7.7]	4 [3–6]	0.974
Visual acuity, worst eye, median [IQR]	0.23 [0.1–0.6]; (n = 18)	0.15 [0.03–0.5]; (n = 8)	0.28 [0.1–0.6]; (n = 10)	0.460
CSF, cell/ μ L, median, [IQR]	8 [2–23.5]; (n = 38);	11 [2.8–18.5]; (n = 18)	7 [2–35.5]; (n = 20)	0.806
Protein concentration, g/L, median [IQR]	0.4 [0.3–0.9]; (n = 37)	0.4 [0.3–0.8]; (n = 17)	0.5 [0.4–0.9]; (n = 20)	0.390
Segments affected on spinal cord MRI, median, [IQR]	3 [2–5]; (n = 37)	3.5 [1.3–6.5]; (n = 20)	3 [2–5]; (n = 17)	0.707
Acute treatment, n (%)				
Iv MP	35 (70)	16 (64)	19 (76)	0.281
PLEX	6 (12)	5 (20)	1 (4)	
Ivlg	1 (2)	0	1 (4)	
Combination therapy	8 (16)	4 (16)	4 (16)	
Chronic treatment after the index event, n (%)	43 (87.8)	23 (92)	20 (87.8)	0.355
GFAP, pg/mL, median, [IQR]	160.5 [87.8–415]	308.3 [146.8–855.9]	103.4 [75.2–202.3]	0.001
NfL, pg/mL, median, [IQR]	18 [7.8–46.5]	26.9 [11.5–52.8]	12.7 [7.2–37.7]	0.113
Tau, pg/mL, median, [IQR]	0.8 [0.5–1.6]	1.2 [0.7–2]	0.51 [0.3–0.8]	0.001
UCH-L1, pg/mL, median, [IQR]	51.5 [28.9–94.2]	61.4 [45.3–133.1]	35 [23.9–70.6]	0.006

Abbreviations: AQP4 = aquaporin 4; DS = double seronegative; EDSS = Expanded Disability Status Scale; GFAP = glial fibrillary acid protein; IQR = interquartile range; Iv MP = IV methylprednisolone; Ivlg = IV immunoglobulin; NfL = neurofilament light chain; NMOSD = neuromyelitis optica spectrum disorder; PLEX = plasma exchange; UCH-L1 = ubiquitin C-terminal hydrolase L1. Results statistically significant ($p < 0.05$) are marked in bold.

Patient Consents

Informed consent for research purposes was obtained from all patients. The study was part of the research protocol approved by the ethics committees of the enrolling centers: prog. 1052CESC Verona-Rovigo approved by the Ethics Committee of Verona University Hospital (Italy); NOMADMUS (OFSEP) registry approved by both the French data protection agency (Commission Nationale de l'Informatique et des Libertés [CNIL]; authorization request 914066v3) and a French ethical committee (Comité de Protection des Personnes [CPP]: reference 2019-A03066-51); the internal project PR(AG)400/2021 approved by the Ethics Committee of Vall d'Hebron Institut de Recerca, Vall d'Hebron University Hospital, Barcelona, Spain; the Ethical Committee of San Luigi Gonzaga University Hospital (approvals number 7262/2019 and 18390/2019); and Mayo Clinic's institutional review board (IRB 08-006647).

Data Availability

Anonymized data not published within this article will be made available on request from any qualified investigator.

Results

Demographic and Clinical Information

The study included 51 adult patients, 25 with AQP4+NMOSD and 26 with DS-NMOSD. The median age at disease onset was 35.1 [IQR 27.4–48] years in patients with AQP4+NMOSD and 39 [24.1–50.5] years in patients with DS-NMOSD ($p = 0.611$). Female sex was more common in both groups (84% in AQP4+NMOSD vs 61.5% in

DS-NMOSD, $p = 0.072$). Clinical presentation at onset was similar in the 2 groups (Table 1).

Samples were collected at onset in 14 cases (27.5%) and during a relapse in 37 patients (72.5%). The median age at sampling was 36.8 [29.4–48.6] years in patients with AQP4+NMOSD and 42.8 [27.6–53.4] years in patients with DS-NMOSD, with a median disease duration of 37.1 [2.4–103.6] and 23 [5.0–99.2] months, respectively, without statistical differences between groups (Table 2). The most common clinical syndromes at sampling in both groups were myelitis (56% in AQP4+NMOSD vs 38.5% in DS-NMOSD) and optic neuritis (32% vs 34.6%, respectively, $p = 0.716$). No statistical differences between the 2 groups were noted for disability at and before sampling, disease course, treatment before and after sampling, extension of spinal cord lesions, and CSF parameters (Table 2).

Follow-up, disease course, final disability, and the number of patients under chronic treatment were not significantly different in the AQP4+NMOSD and DS-NMOSD groups (Table 3).

Serum Biomarker Profile

Median serum GFAP, tau, and UCH-L1 levels were significantly higher in the AQP4+NMOSD group compared with patients with DS-NMOSD (median 308.3 vs 103.4 pg/mL $p = 0.001$; median 1.2 vs 0.5 pg/mL, $p = 0.001$; and median 61.4 vs 35 pg/mL, $p = 0.006$, respectively) while NfL levels were similar ($p = 0.113$; Table 2). Patients under chronic treatment before sampling did not show a different biomarker profile compared with untreated patients (NfL $p = 0.415$, GFAP

Table 3 Disease Course and Outcome

	Whole cohort (n = 51)	AQP4+NMOSD (n = 25)	DS-NMOSD (n = 26)	p Value
Relapsing disease, n (%)	43 (84.3)	20 (80)	23 (88.5)	0.406
N relapse, median, [IQR]	3 [1–5]	3 [1–8]	2 [1–4]	0.411
EDSS at next relapse, median, [IQR]	3.5 [2–6]	2.5 [2–6.9]	4.5 [3–5.5]	0.400
Time from index event to relapse, mo, median [IQR]	10.7 [8.1–34.2]	10.7 [7.1–22.6]	21.1 [7.5–46.6]	0.585
Follow-up, median [IQR]	121.1 [66.7–201.3]	154.7 [68.7–232.9]	100.3 [62.3–146.3]	0.175
EDSS at last follow-up, median [IQR]	2.3 [1–5.6]	3 [1–6.5]	2 [1–4.3]	0.584
Treatment at last follow-up, n (%)				
None	8 (16.3)	2 (8)	6 (25)	0.063
AZT	5 (10.2)	2 (8)	3 (12.5)	
MMF	6 (12.2)	5 (20)	1 (4.2)	
Anti-CD20	22 (44.9)	14 (56)	8 (33.3)	
Tocilizumab	4 (8.2)	2 (8)	2 (8.3)	
Other	4 (8.2)	0	4 (16.7)	

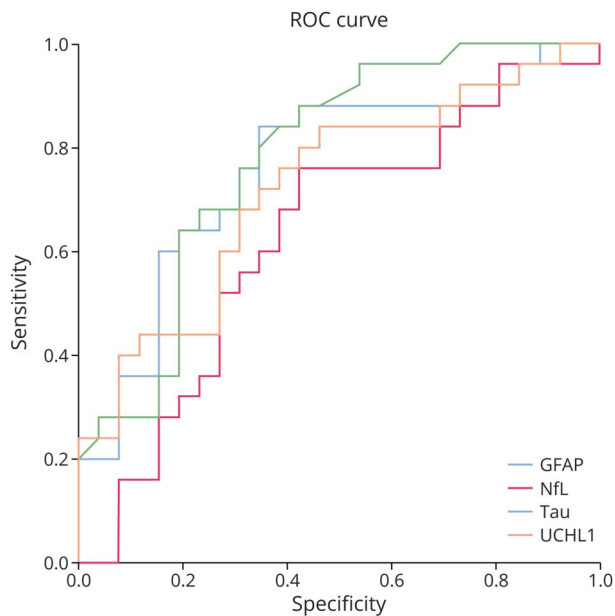
Abbreviations: AQP4 = aquaporin-4; AZT = azathioprine; DS = double seronegative; EDSS = Expanded Disability Status Scale; IQR = interquartile range; MMF = mycophenolate mofetil; NMOSD = neuromyelitis optica spectrum disorder.

$p = 0.361$, $\tau p = 0.602$, UCHL-1 $p = 0.311$). More detailed information about disease course and outcomes is available in Table 3. ROC curve analysis showed that tau and GFAP were the best biomarkers in distinguishing between AQP4+NMOSD and DS-NMOSD (area under the curve (AUC) tau: 0.782, $p = 0.001$, AUC GFAP: 0.762, $p = 0.001$, AUC UCHL-1: 0.723, $p = 0.006$, Figure 1). The tau cutoff was identified at 0.67 pg/mL (sensitivity 0.800, specificity 0.654, accuracy 0.725), the GFAP cutoff at 138.2 pg/mL (sensitivity 0.840, specificity 0.654, accuracy 0.745), and the UCH-L1 cutoff at 44.98 pg/mL (sensitivity 0.760, specificity 0.577, accuracy 0.667).

The biomarker combination profile (UCH-L1, GFAP, and tau; GFAP and tau; and tau and UCH-L1) on ROC curve analysis showed similar discriminative power, with a slightly higher AUC than the single biomarker alone (eAppendix 1, links.lww.com/NXI/A955).

Of note, 2 seronegative patients displayed higher concentration of GFAP, tau, and UCH-L1 values and were independently tested with a live cell-based assay for AQP4-Abs that was confirmed as negative in both cases. The comparison of biomarker profile according to the clinical phenotype is demonstrated in Figure 2.

Figure 1 Receiver-Operating Characteristic (ROC) Curve Representing the Discriminatory Power of Different Biomarkers in Differentiating AQP4+NMOSD and DS-NMOSD



Tau, GFAP, and UCH-L1 were discriminated efficiently between the 2 conditions. Tau: area under curve (AUC) 0.782 (95% confidence interval (CI) 0.656–0.909), $p = 0.001$; GFAP: AUC 0.762 (95% CI 0.627–0.896), $p = 0.001$; UCH-L1: AUC 0.723 (95% CI 0.583–0.864), $p = 0.006$. On the contrary, NfL concentration were not discriminated between seropositive and seronegative NMOSD (AUC: 0.629, 95% CI 0.473–0.786, $p = 0.113$).

Correlation With Attack Clinical Variables and Disease Course

NfL levels were associated with disease severity during the acute phase (EDSS at nadir) in the whole cohort and in the AQP4+NMOSD group. Details of correlation analysis are provided in Table 4.

Discussion

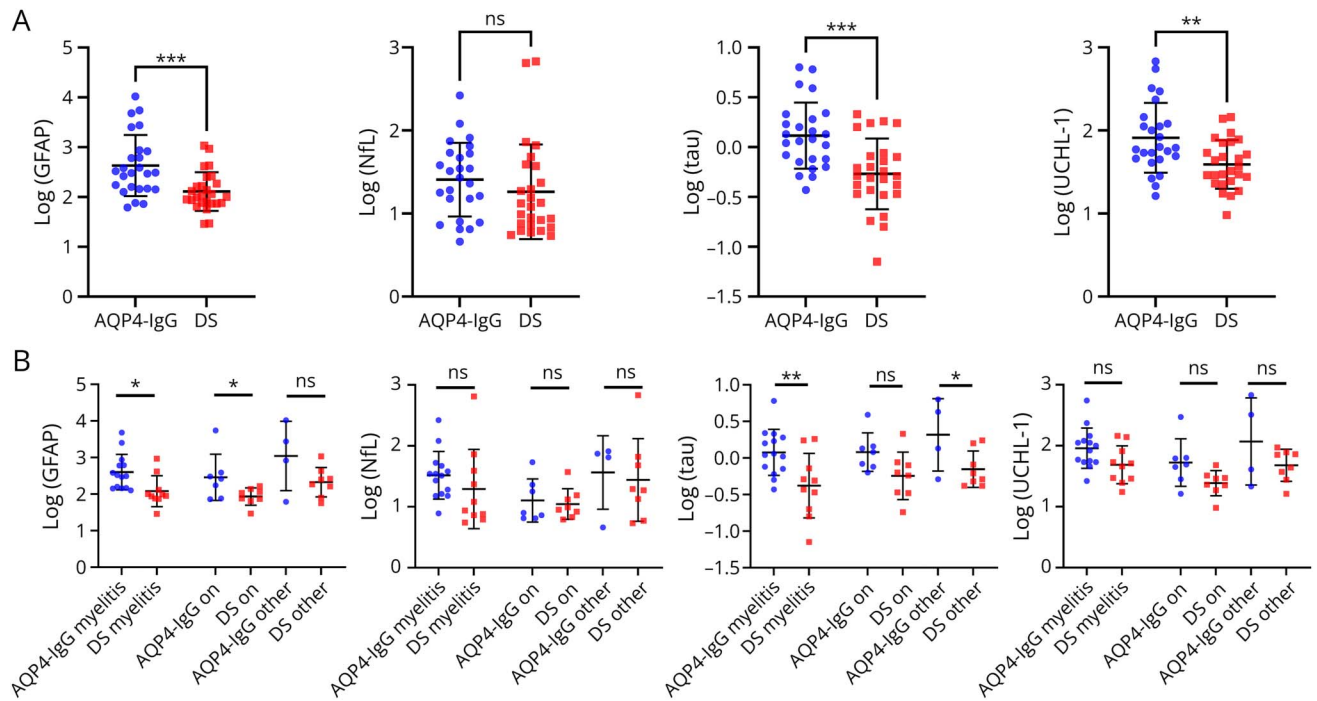
In this retrospective study involving a cohort of patients with AQP4+NMOSD and DS-NMOSD matched per age, with homogenous phenotype and similar disease severity, we found that (1) GFAP, tau, and UCH-L1 levels during the acute phase are significantly higher in patients with AQP4+NMOSD in comparison with those with DS-NMOSD, with GFAP and tau being promising biomarkers in discriminating between these 2 entities, and (2) NfL levels were associated with EDSS at the index event only in patients with AQP4+NMOSD, whereas this association was not observed in DS-NMOSD.

The findings suggesting a different degree of astrocytic damage between AQP4+NMOSD and DS-NMOSD were already reported in the literature on smaller cohorts.^{10,15,20}

The present study confirms on a larger population of DS-NMOSD cases that serum GFAP concentrations are significantly higher in AQP4+ patients, expanding previous similar data reporting lower CSF GFAP levels in DS-NMOSD in comparison with AQP4+NMOSD.²⁰ As a novel finding, tau and UCH-L1 concentration displayed similar results, with tau and GFAP being the best biomarkers to discriminate between AQP4+NMOSD and DS-NMOSD. The different biomarker profile observed in patients with AQP4+NMOSD and DS-NMOSD suggests that the underlying pathophysiology is different, although these diseases share a similar degree of neuronal damage (as expressed by the release of NfL). Of note, a subset of seronegative patients has similar GFAP and UCH-L1 levels to that observed in patients with AQP4+NMOSD, suggesting the possible presence of a still unknown astrocytic target. Further studies, including astrocyte-binding assays, may be useful to identify these antigens. Tau and UCH-L1 have not been extensively investigated as biomarkers in CNS neuroinflammatory disorders. Elevation of tau levels during relapses was previously reported in MOGAD, but not in NMOSD.¹³ Recent studies have suggested that the glymphatic system, whose major driver is AQP4, has a key role in the clearance of tau, with important effects on phosphorylated tau deposition and subsequent neurodegeneration.^{21,22} Increased tau concentration during the attacks can be related to the impairment of its clearance due to AQP4 depletion. Whether this process in patients with AQP4+NMOSD has a long-term role in developing cognitive issues and/or a neurodegeneration still needs to be addressed.

Elevation of UCH-L1 has been reported in amyotrophic lateral sclerosis, whereas it has never been investigated in

Figure 2 Plots of log₁₀-Transformed Values of GFAP, NfL, Tau, and UCH-L1 Levels



(A) The comparison (*t* test) between log(GFAP), $p < 0.001$; log(NfL), $p = 0.312$; log(tau), $p < 0.001$; and log(UCHL-1), $p = 0.003$ in AQP4+NMOSD and DS-NMOSD, respectively. (B) The comparison of biomarkers between AQP4+NMOSD and DS-NMOSD according to the clinical phenotype (unilateral optic neuritis, myelitis, and other less frequent phenotypes). Log(GFAP) and log(tau) were higher in patients with AQP4+NMOSD myelitis ($p = 0.01$ and $p = 0.007$, respectively); log(GFAP) was higher in patients with AQP4+NMOSD unilateral optic neuritis ($p = 0.047$); and log(tau) was higher in the AQP4+ NMOSD with other clinical phenotypes ($p = 0.0482$). In these heterogeneous groups, the following phenotypes were included: DS-NMOSD with bilateral optic neuritis ($n = 1$), AQP4+NMOSD with bilateral optic neuritis ($n = 1$), DS-NMOSD with bilateral optic neuritis with cerebral lesion ($n = 1$), AQP4+NMOSD with myelitis with brainstem involvement ($n = 2$), DS-NMOSD with myelitis with brainstem involvement ($n = 1$), AQP4+NMOSD with optic neuritis and myelitis ($n = 1$), and AQP4+NMOSD with cerebellar involvement ($n = 1$).

neuroinflammatory disorders. UCH-L1 is expressed almost exclusively in the brain, and it is involved in the ubiquitin-proteasome pathway. An inflammatory process implies an increased production of a large variety of protein and often brings to cell death and the creation of cell debris. This could trigger an upregulation of the mechanisms involved in protein degradation, including the ubiquitin-proteasome system.¹⁴ Of note, a cluster of familiar patients with NMOSD in China was found to be associated with a variant in another gene involved in this pathway (USP18).²³ Further studies are needed to confirm our findings and understand whether the proteasome-ubiquitin system could play a role in AQP4+NMOSD.

Our data show that NfL levels correlates with the severity of the attack measured by EDSS in AQP4+NMOSD. Previous studies have shown contrasting findings on this topic,^{8,24} probably because of the different time points in which the analyses were performed. Of note, this relationship was not observed in the DS-NMOSD cohort, further supporting the heterogeneity within this disease.

The main limitations of this study are related to the small sample size and retrospective design of the study, which did not allow to consider additional factors such as subgroup

comparisons, evolution of the biomarker profile over time, treatment effect on biomarkers values, and volumetric lesion comparison between groups. Moreover, healthy controls were not included in the study for comparison. Although biomarker dynamics is not completely clear, we considered a cutoff of 3 months from symptom onset to serum collection, which might have influenced biomarker values. Finally, even if recent studies suggest the utility of MOG-Abs CSF testing in seronegative cases,^{25,26} this analysis was not performed because it was not part of the common clinical practice.

To conclude, serum GFAP, tau, and UCH-L1 levels discriminate between AQP4+NMOSD and DS-NMOSD: The different biomarker profile of DS-NMOSD vs AQP4+NMOSD suggests heterogeneity within the former category and provides useful data to improve our understanding of this disease. The analysis of these biomarkers has several practical implications useful in the clinical practice: (1) could facilitate the identification of patients with AQP4+NMOSD, particularly in cases with inconsistent antibody status; (2) could help to identify among DS-NMOSD cases with a biomarker profile similar to that of patients with AQP4+NMOSD; and (3) could give novel cues on the tissue damage underlying NMOSD. All these aspects are of utmost importance for the administration of newly approved treatments, for patients'

Table 4 Correlation Analysis

	EDSS at nadir of the index event	Visual acuity (worst eye)	EDSS at follow-up	n. of segments affected at spinal cord MRI	CSF—cells/ μ L	CSF—protein concentration	n. of subsequent relapses
Correlation analysis: Whole cohort							
GFAP	Rho 0.376 $p = 0.009$	Rho -0.532 $p = 0.023$	Rho 0.311 $p = 0.028$	Rho 0.213 $p = 0.206$	Rho 0.322 $p = 0.049$	Rho 0.181 $p = 0.284$	Rho -0.148 $p = 0.315$
NfL	Rho 0.605; $p \leq 0.001$	Rho -0.294 $p = 0.237$	Rho 0.418 $p = 0.003$	Rho 0.493 $p = 0.002$	Rho 0.361 $p = 0.026$	Rho 0.170 $p = 0.316$	Rho -0.145 $p = 0.325$
Tau	Rho 0.217 $p = 0.144$	Rho -0.380 $p = 0.120$	Rho 0.034 $p = 0.814$	Rho 0.158 $p = 0.349$	Rho 0.316 $p = 0.053$	Rho 0.126 $p = 0.458$	Rho -0.193 $p = 0.189$
UCH-L1	Rho 0.394 $p = 0.006$	Rho -0.483 $p = 0.042$	Rho 0.242 $p = 0.091$	Rho 0.141 $p = 0.404$	Rho 0.391 $p = 0.015$	Rho 0.204 $p = 0.226$	Rho -0.107 $p = 0.470$
Correlation analysis: AQP4+NMOSD							
GFAP	Rho 0.557 $p = 0.005$	Rho -0.571 $p = 0.139$	Rho 0.192 $p = 0.359$	Rho 0.109 $p = 0.649$	Rho 0.183 $p = 0.466$	Rho 0.122 $p = 0.642$	Rho -0.340 $p = 0.096$
NfL	Rho 0.765; $p \leq 0.001$	Rho -0.310 $p = 0.456$	Rho 0.570 $p = 0.003$	Rho 0.565 $p = 0.009$	Rho 0.286 $p = 0.250$	Rho 0.049 $p = 0.851$	Rho -0.312 $p = 0.129$
Tau	Rho 0.406 $p = 0.049$	Rho -0.323 $p = 0.435$	Rho -0.116 $p = 0.581$	Rho 0.048 $p = 0.841$	Rho 0.341 $p = 0.166$	Rho 0.234 $p = 0.367$	Rho -0.451 $p = 0.024$
UCH-L1	Rho 0.495 $p = 0.014$	Rho -0.238 $p = 0.570$	Rho 0.006 $p = 0.977$	Rho 0.053 $p = 0.824$	Rho 0.221 $p = 0.379$	Rho 0.010 $p = 0.970$	Rho -0.387 $p = 0.056$
Correlation analysis: DS-NMOSD							
GFAP	Rho 0.28 $p = 0.295$	Rho -0.482 $p = 0.159$	Rho 0.212 $p = 0.297$	Rho 0.329 $p = 0.197$	Rho 0.406 $p = 0.075$	Rho 0.295 $p = 0.207$	Rho -0.100 $p = 0.649$
NfL	Rho 0.369 $p = 0.083$	Rho -0.299 $p = 0.402$	Rho 0.220 $p = 0.291$	Rho 0.439 $p = 0.078$	Rho 0.428 $p = 0.060$	Rho 0.202 $p = 0.394$	Rho 0.020 $p = 0.927$
Tau	Rho 0.051 $p = 0.818$	Rho -0.348 $p = 0.325$	Rho -0.023 $p = 0.912$	Rho 0.342 $p = 0.179$	Rho 0.253 $p = 0.282$	Rho 0.151 $p = 0.535$	Rho -0.116 $p = 0.599$
UCH-L1	Rho 0.352 $p = 0.099$	Rho -0.506 $p = 0.136$	Rho 0.351 $p = 0.086$	Rho 0.141 $p = 0.590$	Rho 0.551 $p = 0.012$	Rho 0.456 $p = 0.043$	Rho 0.046 $p = 0.776$

Abbreviations: AQP4 = aquaporin-4; DS = double seronegative; EDSS = Expanded Disability Status Scale; GFAP = glial fibrillary acid protein; NfL = neurofilament light chain; NMOSD = neuromyelitis optica spectrum disorder; UCH-L1 = ubiquitin C-terminal hydrolase L1. Statistically significant (p -value after Bonferroni correction ≤ 0.00179) results are marked in bold.

inclusion in clinical trials, and for the design of novel therapies.

Future prospective studies including larger cohorts of DS-NMOSD cases with longitudinal biomarker monitoring are needed to confirm our findings and assess their utility in predicting disease status/severity and treatment response. These data would be additionally useful in the design of clinical trials dedicated to patients with DS-NMOSD, for which no treatment is currently licensed.

Acknowledgment

Samples and associated data from CRESM were provided by CRESM Biobank, University Hospital San Luigi Gonzaga (Orbassano).

Study Funding

The authors report no targeted funding.

Disclosure

H. Zéphir has no disclosure related to this work. Unrelated to this work, H. Zéphir received consulting fees from Alexion, Horizon Therapeutics, Roche, Merck, Biogen Idec, Sanofi, and Novartis. Georgina Arrambide has received speaking honoraria and compensation for consulting services or participation in advisory boards from Merck, Roche, and Horizon Therapeutics; travel support for scientific meetings from Novartis, Roche, andECTRIMS; is the editor for Europe of the Multiple Sclerosis Journal - Experimental, Translational and Clinical; and is a member of the International Women in Multiple Sclerosis (iWiMS) Network executive committee and of the European Biomarkers in MS (BioMS-eu) Consortium steering committee; J. Villaceros-Álvarez has received a grant from Instituto de Salud Carlos III, Spain, FI21/00282; A. Cobo-Calvo has received a grant from Instituto de Salud Carlos III, Spain, JR19/00007; E.P. Flanagan has served on advisory boards for Alexion, Genentech,

Horizon Therapeutics, and UCB. He has received speaker honoraria from Pharmacy Times. He received royalties from UpToDate. Dr. Flanagan was a site primary investigator in a randomized clinical trial on Inebilizumab in neuromyelitis optica spectrum disorder run by Medimmune/Viela-Bio/Horizon Therapeutics. Dr. Flanagan has received funding from the NIH (R01NS113828). Dr. Flanagan is a member of the medical advisory board of the MOG project. Dr. Flanagan is an editorial board member of the Journal of the Neurological Sciences and Neuroimmunology Reports. A patent has been submitted on DACH1-IgG as a biomarker of paraneoplastic autoimmunity. S. Mariotto received speaker honoraria from Novartis, Biogen, and Sanofi. Go to [Neurology.org/NN](https://www.neurology.org/NN) for full disclosures.

Publication History

Received by *Neurology: Neuroimmunology & Neuroinflammation* September 7, 2023. Accepted in final form October 10, 2023. Submitted and externally peer reviewed. The handling editor was Editor Josep O. Dalmau, MD, PhD, FAAN.

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Appendix (continued)

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Continued

Appendix (continued)

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