

CSF levels of glutamine synthetase and GFAP to explore astrocytic damage in seronegative NMOSD

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ABSTRACT

Objective To explore levels of astrocytopathy in neuromyelitis optica spectrum disorder (NMOSD) by measuring levels of the astrocytic enzyme glutamine synthetase (GS) and glial fibrillary acidic protein (GFAP), an established astrocytic biomarker known to be associated with disease activity in multiple sclerosis.

Methods Cerebrospinal fluid concentrations of GS and GFAP were measured by ELISA in patients with NMOSD (n=39, 28 aquaporin-4 (AQP4)-Ab-seropositive, 3 double-Ab-seronegative, 4 myelin oligodendrocyte glycoprotein (MOG)-Ab-seropositive and 4 AQP4-Ab-seronegative with unknown MOG-Ab-serostatus), multiple sclerosis (MS) (n=69), optic neuritis (n=5) and non-neurological controls (n=37).

Results GFAP and GS concentrations differed significantly across groups (both $p < 0.001$), showing a similar pattern of elevation in patients with AQP4-Ab-seropositive NMOSD. GS and GFAP were significantly correlated, particularly in patients with AQP4-Ab-seropositive NMOSD ($r_s = 0.70$, $p < 0.001$). Interestingly, GFAP levels in some patients with double-Ab-seronegative NMOSD were markedly increased.

Conclusions Our data indicate astrocytic injury occurs in some patients with double-Ab-seronegative NMOSD, which hints at the possible existence of yet undiscovered astrocytic autoimmune targets. We hypothesise that elevated GS and GFAP levels could identify those double-Ab-seronegative patients suitable to undergo in-depth autoimmune screening for astrocytic antibodies.

INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD) refers to a heterogeneous group of immune-mediated central nervous system diseases that share optic neuritis, transverse myelitis and area postrema syndrome as key clinical features.¹

In recent years, understanding of NMOSD has considerably advanced through the identification of two autoimmune targets. The first auto-antibody that was recognised targets aquaporin-4 (AQP4), a water channel which is expressed in astrocytic foot processes.² Accordingly, AQP4 targeted autoimmune activity results in profound astrocytopathy, a feature that is distinct from multiple sclerosis (MS).^{3,4} The subsequently identified myelin-oligodendrocyte glycoprotein (MOG) auto-antibodies are associated

with damage to oligodendrocytes and myelin, but do not cause astrocytic injury.^{5–8}

Identification of these antibodies has facilitated diagnostic procedures, broadened the clinical spectrum and helps guide treatment decisions in NMOSD.^{9,10} However, some patients present with a clinical phenotype that is consistent with NMOSD, but do not express AQP4 or MOG antibodies (Ab).¹¹ The response to immunosuppression in these patients suggests a possible autoimmune pathology, but the autoimmune target remains unknown.¹² Here, we aim to investigate astrocytopathy across the full NMOSD spectrum, including double-Ab-seronegative patients, to gain insight into the pathophysiological processes at play.

Glial fibrillary acidic protein (GFAP), a part of the astrocyte cytoskeleton, is a very useful biomarker when investigating astrocytic damage.¹³ A very substantial increase in cerebrospinal fluid (CSF) GFAP levels during AQP4-Ab-seropositive NMOSD relapses has consistently been reported, and some reports also show low levels of GFAP in MOG-Ab-seropositive disease.^{4,6,7,14–18}

One disadvantage of using GFAP as a biomarker, however, is its poor solubility which limits the sensitivity of the test.¹³ In order to strengthen the laboratory approach for characterising the widening spectrum of autoimmune astrocytopathies, we have developed a novel assay to detect the predominantly astrocytic enzyme glutamine synthetase (GS).¹⁹ In contrast to GFAP, GS is highly soluble, facilitating detection.^{20,21} We hypothesised that complement-mediated damage to astrocytes would release GFAP and GS. Therefore, CSF levels of GS in NMOSD should reveal a similar pattern to what is observed for GFAP.^{13,22}

In this international multicentre collaborative study, we used these two astrocytic biomarkers to explore levels of astrocytopathy across the different subgroups of NMOSD. We demonstrate that both GS and GFAP are elevated in AQP4-Ab-seropositive NMOSD and GFAP is elevated in double-Ab-seronegative NMOSD as well. These data hint at the existence of a yet unidentified astrocytic autoimmune target in a subset of patients with double-Ab-seronegative NMOSD.



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Table 1 Patient demographics and CSF parameters

	Controls	MS	ON	AQP4+NMOSD	MOG+disease	Double-Ab-seronegative NMOSD	Unknown MOG NMOSD	P value
N	37	69	5	28	4	3	4	
Gender F/M (%F)	19/18 (51%)	52/17 (75%)	4/1 (80%)	22/5 (81%*)	3/1 75%	1/2 (33%)	2/2 50%	>0.05†
Mean age (SD)	43.2 (11.1)	42.1 (10.6)	39.8 (9.5)	48.11 (17.5)	25.0 (24.1)	56.0 (4.4)	37.3 (14.0)	>0.05‡
GFAP ng/mL median (IQR)	0.500 (0.825)	0.60 (0.750)	0.20 (0.40)	5.40 (37.85)	0.00 (0.00)	76.7 (46.85)	0.68 (0.08)	<0.001‡
GS µg/L median (IQR)	235.4 (249.0)	329.4 (315.0)	223.4 (131.0)	490.7 (407.2)	246.4 (74.0)	452.0 (309.0)	487.2 (221.2)	<0.001‡

*Gender data missing for one patient with AQP4-Ab-seropositive NMOSD.

†Fisher's exact test.

‡Kruskall-Wallis test for non-parametric data.

AQP4, aquaporin-4; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; ON, optic neuritis.

METHODS

Patients and non-neurological controls

This retrospective study included patients with NMOSD, MS and optic neuritis (ON) from five centres (see online supplementary table S1). Diagnosis of NMOSD was made according to criteria published by Wingerchuk *et al.*¹ We recruited patients with MS who had undergone lumbar puncture as part of diagnostic procedure. Patients with MS were diagnosed according to criteria published by Polman *et al* and all had a long clinical follow-up, during which they displayed a typical disease phenotype.²³ Patients with ON were diagnosed according to criteria published by Petzold *et al*²⁴ and did not fulfil diagnostic criteria for NMOSD or MS.

Age-matched non-neurological controls were selected from a database of patients referred to the Neurology Department in the Radboud University Medical Centre in Nijmegen during the period 2001–2009, who underwent lumbar puncture as part of the diagnostic workup and were confirmed to not have neurological disease. For these patients, all routine CSF parameters were normal.

Approvals and consents

At the time of collection all patients from the Nijmegen centre gave informed consent to lumbar punctures, including later use for scientific purposes but written consent from the patients was legally not required for our analyses. Written informed consent was obtained from all patients from other participating centres.²⁵

CSF samples

CSF samples were collected in polystyrene or polypropylene tubes, centrifuged (5 min, 860g at room temperature) and stored at –80°C. For storage purposes, 20 samples with MS had been moved to storage at –20°C, but not more than 6 months prior to analysis. Patient information was encoded to maintain confidentiality.

GFAP ELISA

GFAP levels were measured using a home-made sandwich ELISA (linear up to 250 µg/L; interassay variation coefficient <14%) as previously described.²⁶

For six French patients with NMOSD, GFAP levels have been published previously and sample volumes were too small to repeat the test.²⁷ Although we excluded these data from analysis, because these data were based on a different method and GFAP levels were not directly comparable, we have displayed these results in online supplementary figure S1. Because of insufficient CSF material, GFAP measurements could not be performed for nine additional subjects (two AQP4-Ab-seropositive, two MOG-Ab-seropositive,

one double-Ab-seronegative NMOSD, two patients with MS and two controls).

GS ELISA

GS levels in CSF were measured using our previously published home-made sandwich ELISA incorporating an acidification and neutralisation step for enhanced detection.¹⁹

AQP4-IgG antibody assay

State-of-the-art cell-based assays were used for AQP4-Ab in the French,²⁸ German,²⁹ Brazilian³⁰ and Spanish cohorts.^{31 32}

MOG-IgG antibody assay

MOG-Ab serostatus was retrospectively identified from chart study. MOG-IgG Ab status was assessed in local laboratories by cell-based assay.

Statistical analysis

Categorical variables were described by counts and percentages, and continuous variables by median and IQRs. Data were analysed using R and RStudio. Distribution of age and gender was tested with the Kruskal-Wallis test and Fisher's exact test, respectively. GS and GFAP levels were compared across groups by the Kruskal-Wallis test. Post hoc analysis was performed with Dunn's test, with p values adjusted for multiple comparisons with the Benjamini-Hochberg method. Correlations were performed by Spearman's rank analysis (r_s =Spearman's rho). Multivariate logistic regressions were used to check for distributions of CSF GS and GFAP across groups with two potential confounding factors, age and gender. In the figures, GFAP has been log transformed after adding 1 ($\log_{10}(\text{GFAP}+1)$) for visualisation purposes. Performance of GFAP and GS in discriminating for both NMOSD status and AQP4-Ab-seropositive NMOSD status were analysed by plotting receiver operating characteristics (ROC) curves and calculating associated area under curve (AUC) with corresponding 95% CI. Optimally effective cut-off values were calculated using the Youden Index.

RESULTS

Subject characteristics

We included 39 patients with NMOSD, of which 28 were AQP4-Ab-seropositive, 4 were MOG-Ab-seropositive, 3 were double-Ab-seronegative and 4 were AQP4-Ab-seronegative but had an unknown MOG-Ab-serostatus. Additionally, 69 patients with MS, 5 patients with ON and 37 non-neurological

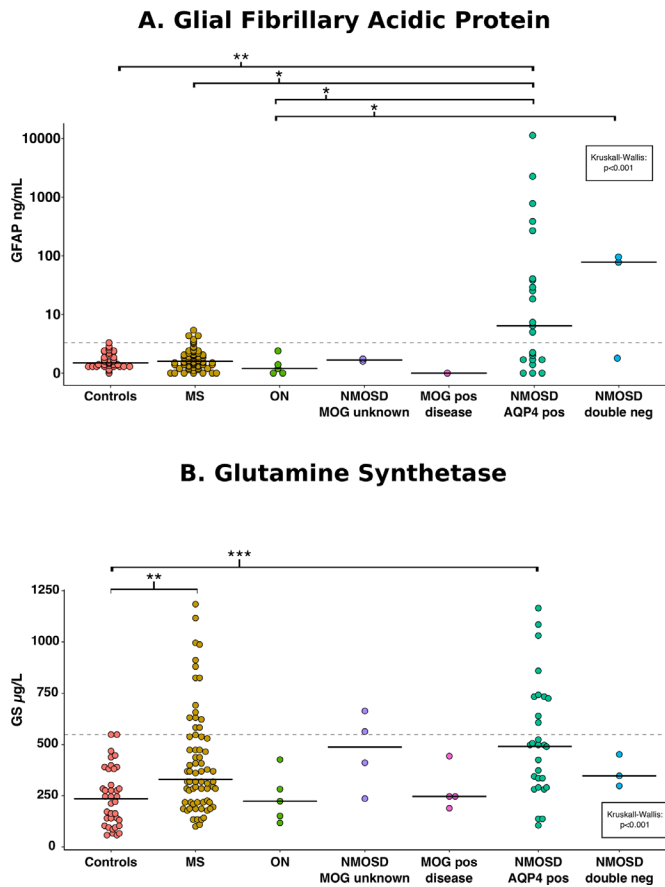


Figure 1 GFAP and GS levels in CSF of controls, MS, ON and NMOSD. (A) CSF GFAP levels in ng/mL of non-neurological control subjects, patients with MS, ON and NMOSD (subgrouped into AQP4-Ab-seropositive, MOG-Ab-seropositive, double-Ab-seronegative and AQP4-Ab-seronegative with unknown MOG-Ab serostatus). The Kruskal-Wallis test showed that there was a statistically significant difference of GFAP levels between groups ($p < 0.01$). The grey dotted line represents the highest measured GFAP concentration in non-neurological control subjects. GFAP has been log transformed after adding 1 ($\log_{10}(\text{GFAP} + 1)$) for visualisation purposes. (B) CSF GS levels in $\mu\text{g/L}$ of non-neurological control subjects, patients with MS, ON and NMOSD (subgrouped into AQP4-Ab-seropositive, MOG-Ab-seropositive, double-Ab-seronegative and AQP4-Ab-seronegative with unknown MOG-Ab serostatus). The Kruskal-Wallis test showed that there was a statistically significant difference of GS between groups ($p < 0.001$). The grey dotted line represents the highest measured GS concentration in non-neurological control subjects. * $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$. AQP4, aquaporin-4; CSF, cerebrospinal fluid; controls, non-neurological control subjects; GFAP, glial fibrillary acidic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; NMO+, AQP4-Ab-seropositive NMOSD, NMO-, AQP4-Ab-seronegative NMOSD; ON, optic neuritis.

control subjects were included (table 1). The baseline characteristics are summarised in table 1. Mean age was comparable between groups ($p = 0.057$). There was a female predominance in the MS and NMOSD groups compared with controls, as is demographically expected, but this difference did not reach significance ($p = 0.051$). For all patients with NMOSD, and for most patients with MS and ON, CSF was obtained acutely during a clinical relapse without concomitant treatment. Detailed CSF and clinical data of the double-Ab-seronegative NMOSD subgroup is given in online supplementary table S2.

Protein biomarkers

Neither GS nor GFAP levels were influenced by the storage conditions (-20°C vs -80°C ; $p > 0.05$).

CSF GFAP levels

Distribution of GFAP levels differed significantly across groups ($p < 0.001$). Median GFAP levels were significantly higher for patients with AQP4-Ab-seropositive NMOSD (5.40 ng/mL) compared with patients with MS (0.60 ng/mL , $p = 0.010$), patients with ON (0.20 ng/mL , $p = 0.014$) and non-neurological controls (0.50 ng/mL , $p = 0.007$).

Interestingly, all GFAP concentrations that were substantially higher than the highest measured in non-neurological controls (2.30 ng/mL) were observed in AQP4-Ab-seropositive NMOSD and double-Ab-seronegative NMOSD (figure 1), although there were six patients with MS with a slightly higher GFAP concentration. Furthermore, median GFAP levels for double-Ab-seronegative patients were significantly increased compared with patients with ON ($p = 0.046$).

Multivariate logistic regression showed that GFAP levels predicted diagnosis of NMOSD ($\text{beta} = 0.461$; $p = 0.0257$) independent of age and gender ($\text{beta} = -0.011$; $p = 0.657$ and $\text{beta} = -0.238$; $p = 0.704$). Additionally, prediction of AQP4-Ab-seropositive NMOSD specifically was nearly significant ($\text{beta} = 0.033$; $p = 0.060$) independent of age and gender ($\text{beta} = 0.034$; $p = 0.163$ and $\text{beta} = -1.309$; $p = 0.096$).

CSF GS levels

Like GFAP, distribution of GS levels differed significantly across groups ($p < 0.001$). GS levels were significantly higher for patients with AQP4-Ab-seropositive NMOSD (median $490.7 \mu\text{g/L}$; $p < 0.001$) and MS (median $329.4 \mu\text{g/L}$, $p = 0.003$) compared with non-neurological controls (median $235.4 \mu\text{g/L}$).

Multivariate logistic regression showed that GS levels predicted diagnosis of NMOSD ($\text{beta} = 0.002$; $p = 0.003$) independent of age and gender ($\text{beta} = 0.025$; $p = 0.098$ and $\text{beta} = -0.034$; $p = 0.939$). Furthermore, GS was a significant predictor of AQP4-Ab-seropositive NMOSD ($\text{beta} = 0.003$; $p < 0.001$) specifically, alongside age ($\text{beta} = 0.052$; $p = 0.006$) but independent of gender ($\text{beta} = -0.436$; $p = 0.442$).

Interestingly, we observed an overall positive correlation between GS and GFAP levels ($r_s = 0.23$, $p < 0.001$), which was particularly strong in AQP4-Ab-seropositive NMOSD ($r_s = 0.70$, $p < 0.001$) but absent for the MS group ($r_s = -0.02$, $p = 0.97$) and controls ($r_s = 0.07$, $p = 0.48$) (figure 2).

Diagnostic performance for astrocytopathy

The AUC of the ROC curves in discriminating for AQP4-Ab-seropositive NMOSD status was 0.75 (95% CI: 0.61 to 0.89) for GFAP and 0.77 (95% CI: 0.67 to 0.86) for GS. The AUC of the ROC curves for identifying all types of NMOSD were slightly lower for both GFAP (0.74 ; 95% CI: 0.61 to 0.86) and GS (0.72 ; 95% CI: 0.63 to 0.82) (figure 3). The optimally effective cut-off of GFAP concentration to identify AQP4-Ab-seropositive NMOSD was 4.0 ng/mL , with an associated specificity of 97% and sensitivity of 57%. The optimally effective cut-off for GS was $268.4 \mu\text{g/L}$, with an associated sensitivity of 89% and specificity of 41%. The negative predictive value of a GS level $< 268.4 \mu\text{g/L}$ was 94%. So, GS can achieve a substantially higher sensitivity but a lower specificity for AQP4-Ab-seropositivity compared with GFAP. This is in line with the observation that all of the 10 patients with AQP4-Ab-seropositive NMOSD who

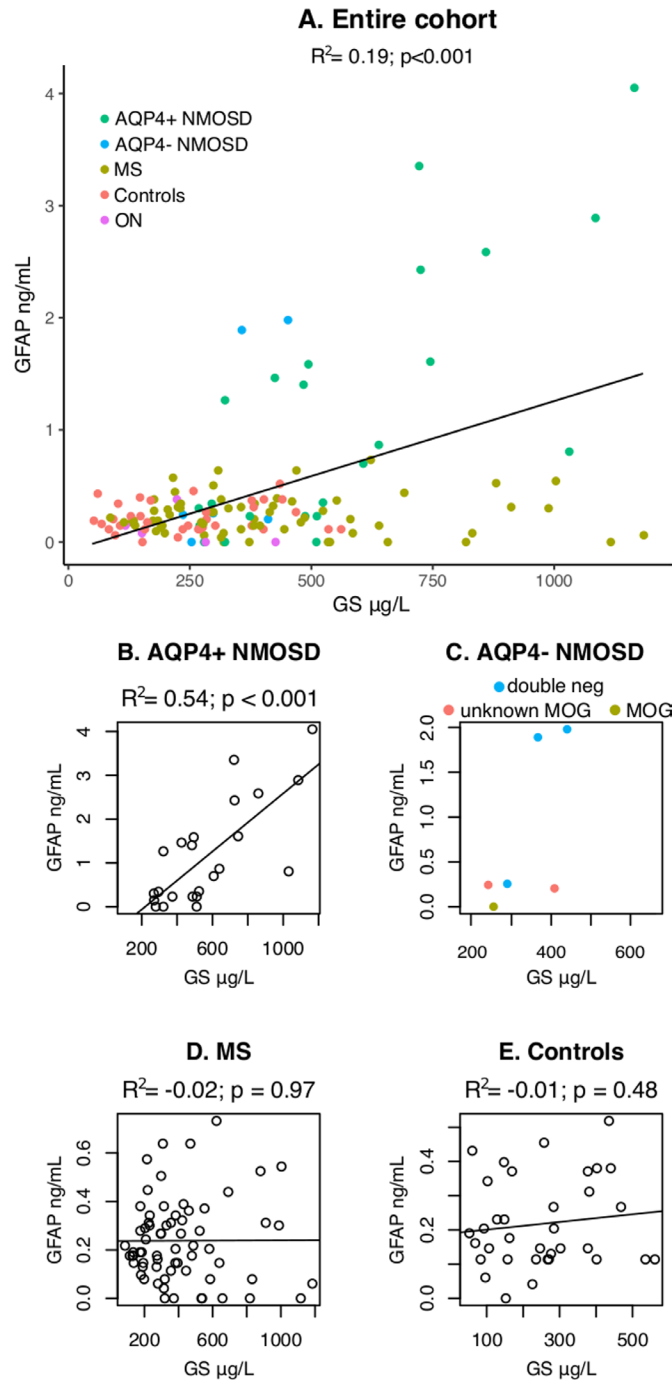


Figure 2 Correlations between GFAP and GS. (A) Linear regression showing significant positive relationship of GFAP and GS in the entire cohort with colour coding of the dots for the different subgroups (AQP4-Ab-seropositive NMOSD, AQP4-Ab-seronegative NMOSD, MS, ON and non-neurological controls). (B) Scatter plot with linear regression line showing the particularly strong positive relationship of GFAP and GS in the AQP4-Ab seropositive NMOSD group. (C) Colour coded scatter plot of GFAP vs GS levels in the double-Ab-seronegative NMOSD, MOG-Ab-seropositive NMOSD and AQP4-Ab-seronegative with unknown MOG-status cases. This plot is shown for visualisation purposes, even though group sizes are too small to draw conclusions or perform correlation analysis. (D) Non-significant relationship between GFAP and GS in the MS cohort. (E) Non-significant relationship between GFAP and GS in the non-neurological control cohort. For all plots GFAP +1 was log transformed for visualisation purposes. AQP4, aquaporin-4; GFAP, glial fibrillar acidic protein; GS, glutamine synthetase; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; ON, optic neuritis.

had GFAP levels below the identified threshold of 4.0 ng/mL, did have GS levels higher than the threshold of 268.4 µg/L.

DISCUSSION

Here, we report CSF levels of astrocytic biomarkers GS and GFAP in NMOSD, MS, ON and non-neurological controls.

Levels of both biomarkers were highest in AQP4-Ab-seropositive NMOSD. The strong correlation between GS and GFAP in AQP4-Ab-seropositive NMOSD suggests that GS is released as a result of astrocytic injury in these patients. GS has a higher sensitivity, but a lower specificity, to astrocytopathy compared with GFAP. Additionally, a subset of double-Ab-seronegative NMOSD

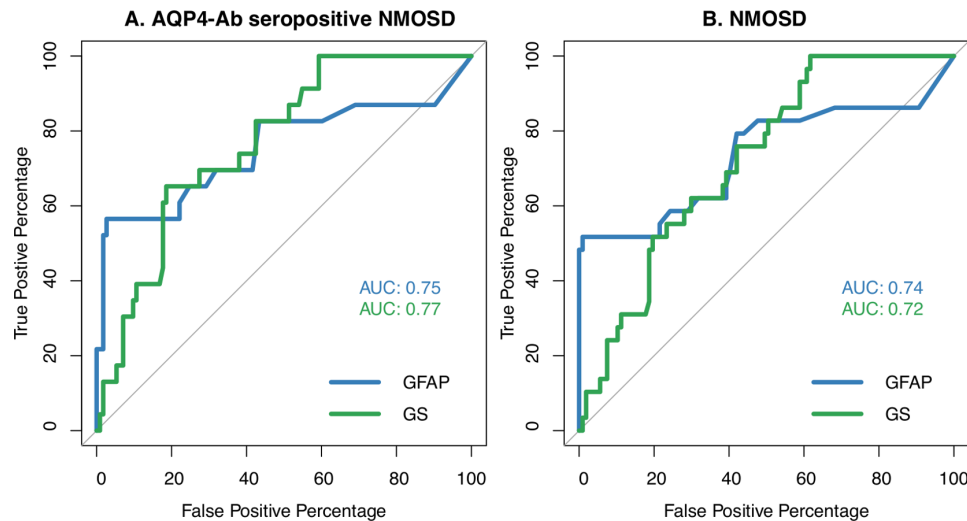


Figure 3 Receiver operating characteristic (ROC) curves GS and GFAP. (A) ROC curve of the discriminative performance for the astrocytopathy AQP4-Ab-seropositive NMOSD of GFAP (blue line) and GS (green line). (B) ROC curve of the discriminative performance for NMOSD of GFAP (blue line) and GS (green line). AQP4, aquaporin-4; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; NMOSD, neuromyelitis optica spectrum disorder.

cases had substantially increased GFAP levels. This observation suggests astrocytic damage in some patients with double-Ab-seronegative NMOSD and hints at the existence of a yet unidentified astrocytic autoimmune target in this group.

Our data replicate prior reports showing that CSF GFAP is increased in AQP4-Ab-seropositive NMOSD but not in MOG-Ab-seropositive NMOSD.^{4,6,7,14–17,33} These results are in line with current understanding of NMOSD pathophysiology, as AQP4-Ab-seropositive NMOSD is an autoimmune astrocytopathy, while MOG-Ab-mediated disease is associated with oligodendrocytic injury but not astrocytopathy.^{3,9} Interestingly, we show that very substantial increases of GFAP are almost exclusive to AQP4-Ab-seropositive NMOSD, but also occur in double-Ab-seronegative NMOSD. This observation appears robust as it has been described before⁴ and we hypothesise that it hints at the existence of one or more yet unidentified auto-antibodies targeting astrocytes in a subset of patients with double-Ab-seronegative NMOSD. A high CSF GFAP concentration may be used to identify those patients with double-Ab-seronegative NMOSD with evidence of astrocytopathy that are suitable for in-depth autoimmune screening using labour intensive clonal expansion techniques. Furthermore, patients with double Ab-seronegative NMOSD pose a substantial diagnostic challenge, given the lack of a reassuring immunological marker.¹ Diagnosis is based solely on clinical and radiological features, resulting in uncertainty when making treatment decisions. A substantially elevated GFAP in these patients with double-Ab negative might reinforce diagnosis of NMO and help guide clinical decision making in some patients.

This is the first study to report on GS levels in NMOSD and related disorders. We showed that CSF levels of this astrocytic enzyme correlate with GFAP levels, especially in AQP4-Ab-seropositive NMOSD, indicating CSF GS may rise as a result of astrocytic damage, as has been suggested previously.²² Furthermore, GS and GFAP levels showed a similar pattern of elevation in AQP4-Ab-seropositive NMOSD, while levels in MOG-Ab-seropositive disease appeared to be generally low. However, in contrast to GFAP, GS levels were elevated in MS compared with control subjects as well. This might be because GS, although primarily an astrocytic enzyme, is expressed by oligodendrocytes to some degree as well.³⁴ Oligodendrocytes are severely

damaged in MS and GS immunoreactivity is reduced in MS brain lesions compared with unaffected and control tissues.³⁵ Additionally, the observed increases of GS in patients with MS and NMOSD could partly arise due to leakage of systemic GS across the blood-brain barrier into the CSF, as the blood-brain barrier is compromised in both disorders. ROC curves of GS and GFAP levels had similar AUCs when testing discriminative performance for AQP4-Ab-seropositive NMOSD. GS provides higher sensitivity compared with GFAP, although specificity for astrocytopathy is relatively low. As GS is elevated more generally in neuroinflammatory disease, it is not an advantageous diagnostic test for NMOSD compared with GFAP. However, GS appears to be a more sensitive marker of astrocytic injury that could be useful to screen for astrocytic injury in patients with seronegative NMOSD that fulfil the stringent clinical diagnostic criteria. The high negative predictive value of 94% associated with GS, suggests that a level below the threshold predicts the absence of astrocytopathy with high accuracy.

The recently described disease entity GFAP autoimmune astrocytopathy may represent the underlying pathophysiological mechanism in some of the double-Ab-seronegative NMOSD with signs of astrocytopathy.³⁶ However, currently there is still some uncertainty if GFAP autoimmunity is a primary disease process or a downstream effect of some forms of astrocytic injury.³⁷ In the future, measuring GFAP in the serum may be a less invasive alternative to CSF measurements, as serum GFAP levels have been shown to be increased in NMOSD as well.¹⁸ An important consideration when talking about double-Ab-seronegative NMOSD diagnosis is the possibility of false-negative AQP4-Ab or MOG-Ab results. In this study, all antibody assessments were based on highly sensitive cell-based assays, minimising risk of false negatives.¹¹

A limitation of this study was the limited available clinical data, especially regarding details on timing of symptom onset relative to CSF acquisition and disability severity, which we know influences GFAP levels.¹³ Furthermore, because CSF volume was limited not all GFAP measurements could be repeated for all patients with the same immunoassay resulting in missing data. Future immunohistochemical analysis of GS and GFAP in patients with NMOSD is needed to confirm the potential role of GS as a CSF biomarker for astrocyte injury

In conclusion, our results indicate there may be one or more yet unidentified astrocytic autoimmune targets in patients with double-Ab-seronegative NMOSD. We propose that screening of patients with double-Ab-seronegative NMOSD for GFAP and GS will identify a subgroup of patients with evidence of astrocytopathy that are suitable for in-depth autoimmune screening to identify a possible new astrocytic immune target. GS has suitable properties for screening purposes, while GFAP can be used as a confirmatory test.

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Contributors MKH, IK and AP were responsible for data collection and statistical analysis, interpretation of the data, the drafting and revision of the manuscript. HBK assisted with the revision of the manuscript and reading the manuscript for intellectual content. DKS, KF, DC, RM, AS, HT, BADJ, SAT and IN were responsible for data collection, assisting with interpretation of the data and reading the manuscript for intellectual content. MMV and AP were responsible for the design and conceptualisation of the study and reading the manuscript for intellectual content and revision of the manuscript. All authors commented on the final version of the manuscript.

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