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--Abseronegative 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 (rs=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 heterogenous group of immune mediated CNS 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 damaged 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 not only release GFAP but also GS. Therefore, CSF levels of GS in NMOSD should reveal a similar pattern to what is observed for GFAP[13,22].

In this international multi-centre 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 double-Ab-seronegative NMOSD. These data hint at the existence of a yet unidentified astrocytic autoimmune target in a subset of double-Ab-seronegative NMOSD patients.

METHODS

Patients and non-neurological controls
This retrospective study included patients with NMOSD, MS and optic neuritis (ON) from five centres (Table S1). Diagnosis of NMOSD was made according to criteria published by Wingerchuk et al. (2015)[1]. We recruited MS patients that had undergone lumbar puncture as part of diagnostic procedure. MS patients were diagnosed according to criteria published by Polman et al. (2005) and all had a long clinical follow-up, during which they displayed a typical disease phenotype[23]. ON patients 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 to 2009, who underwent lumbar puncture as part of the diagnostic work-up and were confirmed to not have neurological disease. For these patients all routine CSF parameters were normal.

Approvals and consents
The CSF samples were collected according to standard protocols with local ethics approval[25]. 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.

CSF samples
CSF samples were collected in polystyrene or polypropylene tubes, centrifuged (5 minutes, 860 g at room temperature), and stored at -80 °C. For storage purposes, 20 MS samples had been moved to storage at -20°C, but not more than six 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 250µg/L; inter-assay 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 Supplementary Figure 1. Because of insufficient CSF material GFAP measurements could not be performed for nine additional subjects (2 AQP4-Ab-seropositive, 2 MOG-Ab-seropositive, 1 double-Ab-seronegative NMOSD, 2 MS patients and 2 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], Brazilian[30] 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 interquartile ranges (IQRs). Data were analysed using R and RStudio. Distribution of age and gender was tested with the Kruskal-Wallis test and Fisher exact test respectively. GS and GFAP levels were compared across groups by the Kruskal-Wallis test. Post-hoc analysis was performed with Dunn 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 (log10(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% confidence interval (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, four were MOG-Ab-seropositive, three were double-Ab-seronegative and four were AQP4-Ab-seronegative but had an unknown MOG-Ab-serostatus. Additionally, 69 patients with MS, five patients with ON and 37 non-neurological 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 to controls, as is demographically expected, but this difference did not reach significance (p=0.051). For all NMOSD patients, and for most MS and ON patients, 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 Table S2.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>MS</th>
<th>Optic Neuritis</th>
<th>AQP+ NMOSD</th>
<th>MOG+ disease</th>
<th>Double-Ab neg NMOSD</th>
<th>Unknown MOG NMOSD</th>
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<td>37</td>
<td>69</td>
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<td>28</td>
<td>4</td>
<td>3</td>
<td>4</td>
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<tr>
<td>Gender F/M (%) (F)</td>
<td>19/18 (51%)</td>
<td>52/17 (75%)</td>
<td>4/1 (80%)</td>
<td>22/5 (81%*)</td>
<td>3/1 75%</td>
<td>1/2 (33%)</td>
<td>2/2 50%</td>
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</tr>
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<td>Mean age (sd)</td>
<td>43.2 (11.1)</td>
<td>42.1 (10.6)</td>
<td>39.8 (9.5)</td>
<td>48.11 (17.5)</td>
<td>25.0 (24.1)</td>
<td>56.0 (4.4)</td>
<td>37.3 (14.0)</td>
<td>&gt;0.05b</td>
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<td>GFAP ng/mL median (IQR)</td>
<td>0.500 (0.825)</td>
<td>0.60 (0.750)</td>
<td>0.20 (0.40)</td>
<td>5.40 (37.85)</td>
<td>0.00 (0.00)</td>
<td>76.7 (46.85)</td>
<td>0.68 (0.08)</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td>GS µg/L median (IQR)</td>
<td>235.4 (249.0)</td>
<td>329.4 (315.0)</td>
<td>223.4 (131.0)</td>
<td>490.7 (407.2)</td>
<td>246.4 (74.0)</td>
<td>452.0 (309.0)</td>
<td>487.2 (221.2)</td>
<td>&lt;0.001b</td>
</tr>
</tbody>
</table>

Table 1. Patient demographics and CSF parameters.

a Fisher exact test. b Kruskall-Wallis test for non-parametric data. * Gender data missing for 1 AQP4-Ab-seropositive NMOSD patient. GFAP: Glial Fibrillary Acidic Protein; GS: Glutamine Synthetase; MS: multiple sclerosis; NMO+: AQP4-Ab-seropositive NMOSD; NMO-: AQP4-Ab-seronegative NMOSD; ON: optic neuritis; IQR: interquartile range.

Protein biomarkers
Neither GS nor GFAP levels were influenced by the storage conditions (-20°C versus -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 MS patients (0.60 ng/mL, p=0.010), ON patients (0.20 ng/mL, p=0.014) and non-neurological controls (0.50 ng/mL, p=0.007).

**Figure 1. GFAP and GS levels in CSF of controls, MS, ON and NMOSD.**

**A. Glial Fibrillary Acidic Protein**

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 (log10(GFAP+1)) for visualisation purposes.

**B. Glutamine Synthetase**

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.

**A:** CSF GFAP levels in ng/mL of non-neurological control subjects, patients with MS, ON and NMOSD (sub-grouped 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 (log10(GFAP+1)) for visualisation purposes.

**B:** CSF GS levels in µg/L of non-neurological control subjects, patients with MS, ON and NMOSD (sub-grouped 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; Controls = non-neurological control subjects; MS = Multiple Sclerosis; NMOSD = neuromyelitis optica spectrum disease; NMO+ = AQP4-Ab-seropositive NMOSD, NMO- = AQP4-Ab-seronegative NMOSD; ON = optic neuritis.

Figure 2. Correlations 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, multiple sclerosis (MS), optic neuritis (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 versus 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 multiple sclerosis (MS) cohort. E. non-
Interestingly, all GFAP concentrations that were substantially higher than the highest measured in non-neurological controls (2.30 ng/mL) were observed exclusively in AQP4-Ab-seropositive NMOSD and double-Ab-seronegative NMOSD (Figure 1), although there were six MS patients with a slightly higher GFAP concentration. Furthermore, median GFAP levels for double-Ab-seronegative patients were significantly increased compared with ON patients (p=0.046).

Multivariate logistic regression showed that GFAP levels predicted diagnosis of NMOSD (Beta=0.461; p=0.0257) independent of age and gender (Beta=-0.011; p=0.657 and Beta=-0.238; p=0.704). Additionally, prediction of AQP4-Ab-seropositive NMOSD specifically was nearly significant (Beta=0.033; p=0.060) independent of age and gender (Beta=0.034; p=0.163 and 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 µg/L; p<0.001) and MS (median 329.4 µg/L, p=0.003) compared with non-neurological controls (median 235.4 µg/L).

Multivariate logistic regression showed that GS levels predicted diagnosis of NMOSD (Beta=0.002; p=0.003) independent of age and gender (Beta=0.025; p=0.098 and Beta=-0.034; p=0.939). Furthermore, GS was a significant predictor of AQP4-Ab-seropositive NMOSD (Beta=0.003; p<0.001) specifically, alongside age (Beta=0.052; p=0.006) but independent of gender (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: 0.61-0.89) for GFAP and 0.77 (95% CI: 0.67-0.86) for GS. The AUC of the ROC curves for identifying all types of NMOSD were slightly lower for both GFAP (0.74; 95% CI: 0.61-0.86) and GS (0.72; 95% CI: 0.63-0.82) (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 µg/L, with an associated sensitivity of 89% and specificity of 41%. The negative predictive value of a GS level lower than 268.4 µg/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 AQP4-Ab-seropositive NMOSD patients that 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 cases had substantially increased GFAP levels. This observation suggests astrocytic damage in some double-Ab-seronegative NMOSD patients 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 results in oligodendrocytic injury but no astrocytopathy[3,9]. Interestingly, we show that very substantial increases of GFAP are not exclusive to AQP4-Ab-seropositive NMOSD, but also occur in double-Ab-seronegative NMOSD. This observation appears robust as it has been described before[4] and we hypothesise that it hints at the existence of one or more yet unidentified auto-antibodies targeting astrocytes in a subset of double-Ab-seronegative NMOSD patients. A high CSF GFAP concentration may be used to identify those double-Ab-seronegative NMOSD patients with evidence of astrocytopathy that are suitable for in-depth autoimmune screening using labour intensive clonal expansion techniques. Furthermore, double Ab-seronegative NMOSD patients 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 double-
Ab negative patients 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[22]. 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 MS and NMOSD patients 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 a more advantageous diagnostic test for NMOSD compared with GFAP. However, GS appears to be a more sensitive marker of astrocytopathy that could be useful to identify astrocytic injury in seronegative NMOSD patients that fulfill the stringent 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 pathophysiologic 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- 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 NMOSD patients 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 double-Ab-seronegative NMOSD patients. We propose that screening of double-Ab-seronegative NMOSD patients 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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AUTHOR CONTRIBUTIONS
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, BAJ, 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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