Granulocyte activation markers in cerebrospinal fluid differentiate acute neuromyelitis spectrum disorder from multiple sclerosis

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ABSTRACT
Background Granulocyte invasion into the brain is a pathological feature differentiating neuromyelitis optica spectrum disorder (NMOSD) from multiple sclerosis (MS). We aimed to determine whether granulocyte activation markers (GAM) in cerebrospinal fluid (CSF) can be used as a biomarker to distinguish NMOSD from MS, and whether levels associate with neurological impairment.

Methods We quantified CSF levels of five GAM (neutrophil elastase, myeloperoxidase, neutrophil gelatinase-associated lipocalin, matrix metalloproteinase-8, tissue inhibitor of metalloproteinase-1) as well as a set of inflammatory and tissue-destuction markers, known to be upregulated in NMOSD and MS (neurolipofilm light chain, glial fibrillary acidic protein, S100B, matrix metalloproteinase-9, intercellular adhesion molecule-1, vascular cellular adhesion molecule-1), in two cohorts of patients with mixed NMOSD and relapsing-remitting multiple sclerosis (RRMS).

Results In acute NMOSD, GAM and adhesion molecules, but not the other markers, were higher than in RRMS and correlated with actual clinical disability scores. Peak GAM levels occurred at the onset of NMOSD attacks, while they were stably low in MS, allowing to differentiate the two diseases for ≤21 days from onset of clinical exacerbation. Composites of GAM provided serum levels of GAM from MS, including all anti-aquaporin-4 protein (aAQP4)-antibody-negative patients who were untreated.

Conclusions GAM composites represent a novel biomarker to reliably differentiate NMOSD from MS, including in aAQP4- NMOSD. The association of GAM with the degree of concurrent neurological impairment provides evidence for their pathogenic role, in turn suggesting them as potential drug targets in acute NMOSD.

WHAT IS ALREADY KNOWN ABOUT THIS TOPIC
⇒ Neuromyelitis optica spectrum disorder (NMOSD) is difficult to differentiate from multiple sclerosis (MS) based on clinical and MRI features, and 20%–40% of patients score negative for the gold-standard diagnostic biomarker, anti-aquaporin-4 protein-antibodies (aAQP4).
⇒ Furthermore, this biomarker does not associate with specific clinical disease features.
⇒ Granulocyte invasion into brain lesions is a key feature of NMOSD, however the potential of granulocyte activation markers (GAM) to differentiate NMOSD from MS has not been explored.

WHAT THIS STUDY ADDS
⇒ Increased cerebrospinal fluid levels of GAM differentiate NMOSD from MS as reliably as aAQP4 in acute stages of disease, including also patients with aAQP4- NMOSD.
⇒ Furthermore, levels of GAM, but not of other biomarkers upregulated in NMOSD, correlate with the actual degree of neurological impairment in acute NMOSD.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ GAM are novel biomarkers for NMOSD that may close a diagnostic gap at the time of first clinical exacerbation, when timely initiation of effective therapy is crucial for therapeutic success.
⇒ In addition, levels of GAM also associate with clinical disability scores at first presentation.
⇒Together with existing preclinical evidence, the current observations suggest a pathogenic role of GAM in NMOSD and may facilitate the development of novel therapeutic approaches for the treatment of acute-stage NMOSD.

INTRODUCTION
Neuromyelitis optica spectrum disorder (NMOSD) and relapsing-remitting multiple sclerosis (RRMS)
share clinical and imaging characteristics, which can make it difficult to differentiate them, and hence may delay the initiation and the choice of adequate therapy. \(^1\) The detection of auto-antibodies targeting the astrocye water channel anti-aquaporin-4 protein (aAQP4) has become a pivotal biomarker tool to diagnose NMOSD. However, 20\%-40\% of patients eventually fulfilling NMOSD criteria remain aAQP4\(^-\) which makes it even more difficult to establish the accurate diagnosis.\(^2\)-\(^5\) Furthermore, the presence or the titre of aAQP4 is not related to clinical disease characteristics.\(^2\) Several biomarkers tend to be more highly elevated in NMOSD than in MS, for example, glial fibrillary acidic protein (GFAP), and S100B (both markers of astrocyte damage),\(^6\) \(^7\) neutrophil light chain light chain (NFL, a marker of neuroaxonal injury),\(^6\) \(^8\) chemokine (C-X-C motif) ligand 13 (CXCL13, a B-cell attractant),\(^9\)-\(^11\) intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) (both leucocyte adhesion molecules)\(^12\) and matrix metalloproteinase-9 (MMP-9, a matrix remodelling gelatinease).\(^13\) Yet, all lack the necessary diagnostic specificity due to overlapping concentration ranges in the two diseases, and the relation between levels in cerebrospinal fluid (CSF) or blood with clinical severity remains uncertain.\(^6\)

In NMOSD, activation of neutrophil granulocytes occurs in blood circulation, and their invasion into inflamed neural tissue is observed in 95\% of NMOSD brain tissue specimens, a feature that differentiates it categorically from typical MS lesions; the involvement of granulocyte invasion in lesion formation has recently been demonstrated also in myelin oligodendrocyte glycoprotein antibody-associated disease (MOGAD).\(^14\)-\(^20\) In the course of acute inflammation, granulocytes release a wide range of proteases and other proteins from their granular compartments, granulocyte activation markers (GAM), some of which are cell-specific such as neutrophil elastase (nEla), myeloperoxidase (MPO), matrix metalloproteinase-8 (MMP-8) and neutrophil gelatinase-associated lipocalin (NGAL), or are partially cell-specific, like tissue inhibitor of metalloproteinase-1 (TIMP-1) and MMP-9. We hypothesised that in NMOSD, including in aAQP4\(^-\) cases, GAM produce a humoral footprint in CSF that allows to differentiate NMOSD from RRMS, and that their levels correlate with clinical severity at the time of CSF sampling to give support for their pathogenetic role in NMOSD, as suggested in preclinical models and neuropathological findings.\(^16\) In this case-control study, we quantified levels of GAM in acute and subacute/chronic (s/c) NMOSD and RRMS, along with MMP-9, NFL, GFAP, S100B, ICAM-1, VCAM-1, CXCL13 to define a precision medicine tool for the diagnosis of acute-stage NMOSD.

### METHODS

**Participants and samples**

The diagnosis of NMOSD with/without aAQP4, and of RRMS was based on respective standard diagnostic criteria.\(^21\) \(^22\) Acute disease exacerbation/relapse was defined according to 2017 McDonald criteria.\(^22\) Disability was assessed using the Expanded Disability Status Scale (EDSS).\(^23\) The discovery cohort from Kyushu University Hospital (Fukuoka, Japan) consisted of 34 patients with NMOSD with 42 CSF samples (2 patients contributed 3 CSF samples, 4 patients contributed 2 CSF samples), and 36 patients with RRMS with 40 CSF samples (4 patients contributed 2 CSF samples); these repeated lumbar punctures were performed following independent disease exacerbations. The validation cohort consisted of 25 patients with NMOSD from Kyushu University Hospital (n=11), Ospedale San Raffaele and Mondino Foundation (Milan and Pavia, Italy) (n=8) and Karolinska University Hospital (Stockholm, Sweden) (n=6) and 46 patients with MS (Kyushu n=18, Karolinska n=28). Two control groups (University Hospital Basel, Switzerland)

### Table 1  Analytical panel of biomarkers

<table>
<thead>
<tr>
<th>Function/Biomarker significance</th>
<th>Analytical platform</th>
<th>LOD (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteolytic enzyme</td>
<td>EnzChek Elastase Assay Kit (ThermoFisher)</td>
<td>18.2</td>
</tr>
<tr>
<td>Hypochlorous acid (HOCI) production</td>
<td>SP-X platform (Simoa) (Quanterix)</td>
<td>0.01</td>
</tr>
<tr>
<td>MMPs: extracellular matrix modulation</td>
<td>SPE-X platform (Simoa) (Quanterix)</td>
<td>0.65</td>
</tr>
<tr>
<td>NGALS: bacteriostatic by Fe chelation</td>
<td>Quantikine ELISA (R&amp;D Systems)</td>
<td>0.33</td>
</tr>
<tr>
<td>MMP activation and inhibition</td>
<td>SP-X platform (Simoa) (Quanterix)</td>
<td>0.74</td>
</tr>
<tr>
<td>Cytoplasmatic calcium-binding protein marker</td>
<td>ELISA (BiosVendor)</td>
<td>3.77</td>
</tr>
<tr>
<td>Cytoskeleton intermediate filament, cell damage marker</td>
<td>HD-X platform (Simoa) (Quanterix)</td>
<td>0.21</td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>SP-X platform (Simoa) (Quanterix)</td>
<td>1.12</td>
</tr>
<tr>
<td>B-cell chemoattractant</td>
<td></td>
<td>0.42</td>
</tr>
</tbody>
</table>

Values below LOD were imputed as a random value between 0.001 and LOD, drawn from a uniform distribution.

* Nomenclature according to https://enzyme.expasy.org.
† Forms complexes with MMP-9; the only other cellular source is from renal tissue.
‡ Other than the name indicates, in granulocytes TIMP-1 is involved in MMP activation.\(^40\)

CNS, central nervous system; CSF, cerebrospinal fluid; LOD, limit of detection; Simoa, single molecule array.
were used to determine values under physiological and highly inflammatory conditions, respectively: ‘symptomatic controls’ (SC)\(^2\) consisted of 25 patients in whom a structural neurological disease was excluded, based on normal findings in clinical MRI evaluations, normal CSF cell composition and protein content and absence of signs of intrathecal immunoglobulin synthesis. The second control group comprised 15 patients with various types of acute inflammatory neurological disease controls\(^2\) (inflammatory neurological disease controls: meningoencephalitis/polyradiculitis due to (a) varicella zoster virus (n=5) and (b) tuberculosis (n=1), (c) viral meningitis (n=3), (d) neuroborreliosis (n=3), (e) eosinophilic encephalitis (n=1), (f) autoimmune encephalitis/myelomingenoradiculitis of unknown cause (n=2)).

**Measurement of biomarkers**

Standard CSF analyses were performed at each centre independently, while here investigated biomarkers were analysed centrally. Expression levels of 12 markers were determined by ELISA and single molecule array assay in the discovery cohort (Table 1). CSF samples with >1 erythrocyte/µL were excluded from the analysis. Sample identities in both cohorts were blinded until all analyses had been completed. All samples and calibrators were assayed in duplicate. An explorative analysis of biomarker levels categorised for sites of origin of samples showed comparable values of GAM (not shown).

**STATISTICAL ANALYSIS**

CSF levels of biomarkers are presented as median and IQRs by diagnostic groups and were compared using the Wilcoxon rank-sum test. To determine the capacity of distinguishing between NMOSD and RRMS without the potential confounding effect of corticosteroid pretreatment, we repeated the same analyses in treatment-naïve patients. To investigate the temporal dynamics of biomarker concentrations, we used for each biomarker an individual linear model to describe the levels in NMOSD and RRMS within a 60-day period after acute disease exacerbation; this period was defined in days between the onset of acute disease exacerbation and lumbar puncture. Biomarker levels were log-transformed and served as dependent variable. Diagnosis (RRMS vs NMOSD) and time since disease exacerbation were used as independent variables. The interaction indicates whether the temporal dynamics differ between patients with NMOSD and RRMS within a 60-day period after acute disease exacerbation; this period was defined in days between the onset of acute disease exacerbation and lumbar puncture; respective analysis included those patients with NMOSD and RRMS who contributed single CSF sample from independent disease exacerbations (see ‘Methods’ section).
<table>
<thead>
<tr>
<th>Cohort</th>
<th>Discovery</th>
<th>NMOSD All (42)*</th>
<th>RRMS All (40)*</th>
<th>P value</th>
<th>NMOSD Acute (20)</th>
<th>NMOSD s/c (22)</th>
<th>P value</th>
<th>NMOSD Acute (17)</th>
<th>RRMS All (46)</th>
<th>P value</th>
<th>RRMS Acute (34)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nEla</td>
<td>42.0</td>
<td>(15.6, 162.6)</td>
<td>15.3</td>
<td>0.001</td>
<td>114.8 (44.9, 670.8)</td>
<td>17.1 (10.2, 42.3)</td>
<td>&lt;0.001</td>
<td>15.2 (8.3, 38.9)</td>
<td>220.7 (83.7, 435.2)</td>
<td>&lt;0.001</td>
<td>220.7 (14.4, 435.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>MPO</td>
<td>3.8</td>
<td>(0.7, 10.7)</td>
<td>1.2</td>
<td>0.013</td>
<td>7.9 (2.8, 73.7)</td>
<td>1.2 (0.3, 5.2)</td>
<td>0.003</td>
<td>0.6 (0.2, 3.4)</td>
<td>253.7 (0.0, 706.8)</td>
<td>0.016</td>
<td>280.9 (0.0, 731.7)</td>
<td>0.006</td>
</tr>
<tr>
<td>NGAL</td>
<td>5528</td>
<td>(2846, 7877)</td>
<td>3809</td>
<td>0.001</td>
<td>6411 (5173, 17148)</td>
<td>4021 (2292, 6302)</td>
<td>0.010</td>
<td>2210 (2087, 4374)</td>
<td>3289 (1762, 4642)</td>
<td>&lt;0.001</td>
<td>3786 (2054, 5263)</td>
<td>0.004</td>
</tr>
<tr>
<td>MMP-8</td>
<td>3.6</td>
<td>(2.5, 9.0)</td>
<td>2.5</td>
<td>0.004</td>
<td>8.7 (3.3, 127.3)</td>
<td>3.1 (1.9, 4.6)</td>
<td>0.005</td>
<td>2.6 (1.8, 25.1)</td>
<td>6.2 (2.7, 1.3)</td>
<td>&lt;0.001</td>
<td>6.2 (2.2, 1.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MMP-9</td>
<td>136.1</td>
<td>(23.9, 1923)</td>
<td>101.6</td>
<td>0.400</td>
<td>270.2 (37.3, 2199)</td>
<td>369 (18.6, 1600)</td>
<td>0.265</td>
<td>174.3 (26.5, 1031)</td>
<td>1732 (1168, 5156)</td>
<td>0.981</td>
<td>1732 (1135, 3967)</td>
<td>0.493</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>55.9</td>
<td>(44.1, 85.7)</td>
<td>39.2</td>
<td>&lt;0.001</td>
<td>62.7 (51.2, 155.8)</td>
<td>51.6 (41.4, 61.6)</td>
<td>0.032</td>
<td>38.0 (34.3, 39.6)</td>
<td>24.2 (18.3, 42.8)</td>
<td>&lt;0.001</td>
<td>28.8 (21.6, 47.7)</td>
<td>0.011</td>
</tr>
<tr>
<td>GFAP</td>
<td>14.6</td>
<td>(8.1, 39.3)</td>
<td>7.5</td>
<td>&lt;0.001</td>
<td>3.0 (10.7, 611.2)</td>
<td>12.2 (7.4, 19.4)</td>
<td>0.060</td>
<td>7485 (5541, 11321)</td>
<td>1683 (153.1, 1883)</td>
<td>0.005</td>
<td>295.3 (192.4, 448.6)</td>
<td>0.030</td>
</tr>
<tr>
<td>S100B</td>
<td>219.6</td>
<td>(166.5, 311.9)</td>
<td>175.8</td>
<td>0.009</td>
<td>249.2 (186.0, 496.6)</td>
<td>199.7 (155.4, 246.6)</td>
<td>0.030</td>
<td>168.3 (153.1, 1883)</td>
<td>291.8 (192.4, 448.6)</td>
<td>0.003</td>
<td>295.3 (192.4, 448.6)</td>
<td>0.052</td>
</tr>
<tr>
<td>NL</td>
<td>1739</td>
<td>(1168, 5156)</td>
<td>1980</td>
<td>0.981</td>
<td>1732 (1135, 3967)</td>
<td>2653 (1254, 6129)</td>
<td>0.371</td>
<td>2436 (1397, 8061)</td>
<td>1732 (1135, 3967)</td>
<td>0.981</td>
<td>1732 (1135, 3967)</td>
<td>0.493</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>3198</td>
<td>(1968, 4196)</td>
<td>2008</td>
<td>&lt;0.001</td>
<td>3698 (2005, 6225)</td>
<td>2562 (1880, 3837)</td>
<td>0.116</td>
<td>1566 (1720, 2560)</td>
<td>1729 (1300, 2582)</td>
<td>0.002</td>
<td>1286 (957.6, 1693)</td>
<td>0.011</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>252.2</td>
<td>(202.4, 362.4)</td>
<td>201.3</td>
<td>0.001</td>
<td>266.9 (226.2, 464.2)</td>
<td>230.0 (184.4, 289.5)</td>
<td>0.087</td>
<td>219.8 (173.5, 245.2)</td>
<td>160.4 (132.5, 267.5)</td>
<td>0.010</td>
<td>167.5 (140.6, 281.1)</td>
<td>0.004</td>
</tr>
<tr>
<td>CXCL13</td>
<td>7.4</td>
<td>(2.5, 41.2)</td>
<td>4.8</td>
<td>0.228</td>
<td>11.4 (3.9, 47.9)</td>
<td>3.4 (2.0, 29.2)</td>
<td>0.149</td>
<td>6.7 (2.9, 21.5)</td>
<td>1.3 (2.7, 3.2)</td>
<td>0.326</td>
<td>2.5 (2.0, 34.2)</td>
<td>0.120</td>
</tr>
</tbody>
</table>

Values are medians (IQR) in pg/mL for nEla, MPO, NGAL, MMP-8, MMP-9, S100B, NF, and ICAM-1, and in pg/mL×10³ for TIMP-1, GFAP, and VCAM-1. P values ≤0.05 are with green background, p values 0.05–0.10 are with yellow background. The analysis without CSF samples from repetitive relapses (NMOSD: n=34, RRMS: n=36) yielded the same statistical differences as shown in table 3, except for MPO (p=0.097) and S100B (p=0.083).*Six patients with NMOSD and four patients with RRMS contributed >1 CSF sample from independent disease exacerbations (see ‘Methods’ section).†P for comparison of acute NMOSD and four patients with RRMS contributed >1 CSF sample from independent disease exacerbations (see ‘Methods’ section).
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NMOSD and RRMS. Again, sensitivity analyses were run after exclusion of pretreated patients. In accordance with the exploratory nature of these analyses, no correction for multiple testing was performed. Accordingly, p values should not be interpreted as confirmatory but rather as a continuous measure of evidence against the corresponding null-hypothesis.

The correlation between biomarker levels and EDSS score was quantified using Spearman’s rank correlation coefficient. The diagnostic capacity of GAM to differentiate NMOSD from RRMS in acute stages (≤21 days after onset of exacerbations) was determined by a logistic model where the disease type (NMOSD vs RRMS) served as dependent variable, and biomarkers, or composites of biomarkers, as independent variables. The predictions from these models were assessed with receiver operating characteristic (ROC) curves, based on pooled data of discovery and validation cohorts. We performed this analysis with and without time of sampling since disease exacerbation as a covariate, in acute patients and in those without corticosteroid pretreatment. For each model, the area under the curve (AUC), as well as sensitivity, specificity, positive and negative predictive value, based on the optimal cut-off according to the Youden Index, are presented. To test the robustness of the model in terms of replicability and to address the risk of overfitting in function of the numbers of markers and time as a covariate in composite models, we validated them by calculating optimism-corrected AUCs based on 500 bootstrap replicates. All analyses were carried out using the statistical software R (V.4.1.2, The R Foundation for Statistical Computing). The significance level was set at p=0.05.

RESULTS

Demographics of discovery and validation cohort of patients

Table 2 shows the baseline characteristics of patients with NMOSD and RRMS in the discovery and validation cohort; they were stratified into ‘acute’ (≤21 days) and s/c (>21 days) stages, depending on the time between onset of acute clinical symptoms and lumbar puncture. All patients with NMOSD were aAQP4+, except three in the discovery and four in the validation cohort (of those, one became positive during a later attack); of these seven patients, six scored negative for anti-MOG antibodies (one patient was not tested). Patients with NMOSD in both cohorts were older and had higher EDSS scores than patients with RRMS. The majority of CSF samples (76.2% in the discovery and 48.0% in the validation cohort) were from patients with NMOSD on continuous oral, or who had received intravenous corticosteroid therapy before the time of CSF sampling, while for patients with RRMS the corresponding proportions were much smaller (37.5% and 8.7%, respectively).

Biomarker expression profiles in NMOSD and RRMS in discovery and validation cohorts

In the discovery cohort, GAM levels were higher in (a) NMOSD versus RRMS overall, (b) acute versus s/c NMOSD and (c) acute NMOSD versus acute RRMS. The astrocyte markers GFAP and S100B and adhesion molecules VCAM-1 and ICAM-1 were increased in NMOSD versus RRMS (overall and acute), while in acute versus s/c NMOSD this was only the case for S100B. In
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In acute NMOSD, the median expression levels of granulocyte-specific GAM (nEla, MPO, NGAL) and of TIMP-1 were similar compared with INCDS; only MMP-8 was slightly higher in NMOSD versus INDC. All analysed markers were higher in acute NMOSD versus SC except for S100B (online supplemental table 1). In s/c NMOSD, all GAM markers, TIMP-1, ICAM-1 and VCAM-1 were lower compared with INDC (online supplemental figure 1A).

Because many markers analysed in the discovery cohort have not been evaluated in NMOSD, we decided to confirm these results in an independent validation cohort. The findings for GAM were fully confirmed in the validation cohort for the comparison of NMOSD versus RRMS (both ‘all’ and ‘acute’), and in part for the comparison of acute versus s/c NMOSD (only four s/c NMOSD samples available) (table 3). Subsequent analyses were therefore performed in the merged discovery/validation set.

Other than in NMOSD, there were no significant differences between acute and s/c levels of GAM and the other markers in RRMS, apart from NGAL and TIMP-1 being higher in s/c RRMS (p=0.013 and p=0.006, respectively), while all other markers were not different between these disease stages in the merged discovery/validation set.

Impact on biomarker levels by immunomodulatory and corticosteroid therapy prior lumbar puncture

GAM levels of patients under immunomodulatory plus corticosteroid therapy showed a strong overlap compared with those of patients being treated only with corticosteroids, in both acute and s/c phases, suggesting that these compounds used for prevention of further NMOSD relapses have no significant impact on granulocyte activation; in contrast, patients under corticosteroid therapy had lower GAM levels in s/c, and to a lesser extent in acute NMOSD, compared with patients without treatment (online supplemental figure 1B). However, GAM and adhesion molecule levels were only numerically higher without as compared with the combined groups with corticosteroid (overall and intravenous); only for nEla this was significant (online supplemental table 2).

After exclusion of corticosteroid treated patients, the higher levels of GAM and adhesion molecules in NMOSD versus contrast, levels of NfL, MMP-9 and CXCL13 were not different for these three comparisons (table 3, online supplemental figure 1A).

Figure 2 Modelled kinetics of biomarker levels in NMOSD and RRMS in function of days after disease exacerbation. Biomarker values are in pg/mL. Values on x-axis show days after disease exacerbations. Dotted lines determine 95% CI, based on all patients. (A) Pattern 1: increased in NMOSD, stably low (nEla, MPO, MMP-8, GFAP, S100B, ICAM-1, VCAM-1), or slightly increasing over time (NGAL, TIMP-1) in RRMS. (B) Pattern 2: increased in both NMOSD and RRMS at disease exacerbation: MMP-9 and CXCL13; pattern 3: stably high in NMOSD and RRMS: NfL. NMOSD: yellow (pooled cohorts), red (discovery cohort only); RRMS: brown (pooled cohorts), green (discovery cohort only). CXCL13, C-X-C motif chemokine 13; GFAP, glial fibrillar acidic protein; ICAM-1, intercellular adhesion molecule-1; MMP, matrix metalloproteinase; MPO, myeloperoxidase; nEla, neutrophil elastase; NGAL, neutrophil gelatinase-associated lipocalin; NfL, neurofilament light chain; NMOSD, neuromyelitis optica spectrum disorder; RRMS, relapsing-remitting multiple sclerosis; TIMP-1, tissue inhibitor of metalloproteinase-1; S100B, S100 calcium-binding protein; VCAM-1, vascular cell adhesion molecule-1.
Association between biomarker levels disease severity/disability status, aAQP4 status and CSF granulocyte count

Table 4 ROC analyses of pattern 1 biomarkers (granulocyte-activation markers, S100B, adhesion molecules) to differentiate NMOSD from RRMS of pooled cohorts in acute stages in patients without corticosteroid pretreatment

<table>
<thead>
<tr>
<th>Marker</th>
<th>AUC</th>
<th>Time</th>
<th>Optimism corrected</th>
<th>Youden Index</th>
<th>Specificity</th>
<th>Sensitivity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>nEla, MPO, MMP-8</td>
<td>–</td>
<td>0.69 (0.49 to 0.89)</td>
<td>0.61</td>
<td>0.35</td>
<td>0.95</td>
<td>0.53</td>
<td>0.80</td>
<td>0.85</td>
</tr>
<tr>
<td>+</td>
<td>0.61 (0.42 to 0.78)</td>
<td>0.60</td>
<td>0.34</td>
<td>0.98</td>
<td>0.54</td>
<td>0.88</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>–</td>
<td>0.69 (0.51 to 0.88)</td>
<td>0.62</td>
<td>0.42</td>
<td>0.98</td>
<td>0.47</td>
<td>0.88</td>
<td>0.84</td>
</tr>
<tr>
<td>+</td>
<td>0.76 (0.60 to 0.92)</td>
<td>0.63</td>
<td>0.34</td>
<td>0.95</td>
<td>0.46</td>
<td>0.75</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td>–</td>
<td>0.71 (0.54 to 0.89)</td>
<td>0.66</td>
<td>0.34</td>
<td>0.86</td>
<td>0.53</td>
<td>0.57</td>
<td>0.84</td>
</tr>
<tr>
<td>+</td>
<td>0.74 (0.58 to 0.90)</td>
<td>0.63</td>
<td>0.28</td>
<td>0.79</td>
<td>0.62</td>
<td>0.47</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>nEla, NGAL, MPO, MMP-8, composite 1</td>
<td>–</td>
<td>0.70 (0.59 to 0.80)</td>
<td>0.84</td>
<td>0.22</td>
<td>0.81</td>
<td>0.87</td>
<td>0.62</td>
<td>0.94</td>
</tr>
<tr>
<td>+</td>
<td>0.96 (0.60 to 1.00)</td>
<td>0.75</td>
<td>0.08</td>
<td>0.76</td>
<td>1.00</td>
<td>0.57</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>nEla, NGAL, MPO, MMP-8, TIMP-1; composite 2</td>
<td>–</td>
<td>0.94 (0.80 to 1.00)</td>
<td>0.89</td>
<td>0.52</td>
<td>1.00</td>
<td>0.87</td>
<td>1.00</td>
<td>0.95</td>
</tr>
<tr>
<td>+</td>
<td>0.98 (0.94 to 1.00)</td>
<td>0.79</td>
<td>0.48</td>
<td>1.00</td>
<td>0.92</td>
<td>1.00</td>
<td>0.98</td>
<td></td>
</tr>
</tbody>
</table>

RRMS (‘all’ and ‘acute’) as seen in overall patients (table 3) were confirmed, while those of MMP-9, NfL and CXCL13 were again not different; this was also the case for GFAP (not shown).

Temporal dynamics of biomarker levels in relation to time between disease exacerbation and lumbar puncture

To further explore the temporal dynamics of biomarker levels observed by categorical analysis (table 3), we ran a time-dependent model applying a time window of up to 60 days after disease exacerbation (figure 2A,B). Thus, we identified three different kinetic patterns of biomarkers in NMOSD versus RRMS. Pattern 1, characterised by peak levels at NMOSD disease exacerbation with stably low or only slightly increased levels (NGAL, TIMP-1) in RRMS, comprised GAM, GFAP, S100B and adhesion molecules. All these markers discriminated NMOSD from RRMS based on the non-overlapping pointwise 95% CIs within the acute disease stage, that is, ≤21 days after disease exacerbations. In contrast, MMP-9, CXCL13 and NfL were strongly upregulated in NMOSD (online supplemental table 3). Being substrates for proteolytic cleavage from the cell surface by nEla and other granulocyte proteases, levels of nEla showed a strong correlation with those of ICAM-1 and VCAM-1, while this was not the case for RRMS (online supplemental figure 2); results remained essentially the same when corticosteroid-treated patients were excluded (not shown).

Granulocytes were present in nine (21%) CSF samples of patients with NMOSD of the discovery cohort. The granulocyte CSF cell count showed strong correlation with levels of granulocyte-specific activation markers, while there was only a trend for TIMP-1 and MMP-9, and no correlation with NfL and CXCL13 (online supplemental figure 3). None of these markers correlated with the CSF granulocyte cell count in INDCs or in RRMS.
Neuro-inflammation

Efficacy of single and combined biomarkers to differentiate between acute stages of NMOSD and RRMS

We next explored the diagnostic value of single GAM concentrations and of their composites to differentiate NMOSD from RRMS. To simulate a situation of unclear differential diagnosis at first disease exacerbation, we restricted the analyses to patients in acute disease stage who had not been exposed to corticosteroids before CSF sampling. Results were expressed as ROC curves with analyses being performed with and without time since exacerbation as covariate. Introducing the time elapsed from symptom onset to CSF sampling as cofactor did not improve AUC values of single GAM (0.74–0.91 with, and 0.69–0.85 without time as covariate), and had an inconsistent effect on measures of prediction of diagnosis (table 4). The combination of granulocyte-specific GAM (composite 1) alone, or in addition with TIMP-1 (composite 2), as an integrated marker raised AUC values to levels 0.90 and 0.94, respectively, leading to sensitivity and specificity values of 0.87 and 0.81 (composite 1) and 0.87 and 1.0 (composite 2), respectively (figure 3, table 4). Here, the inclusion of time as covariate further improved specificity and sensitivity values of composite 2 to 1.00 and 0.92. Neither the additional inclusion of S100B nor that of adhesion molecules into a larger composite improved the capacity

Figure 3  ROC curves for the differentiation between NMOSD and RRMS in patients without corticosteroid pretreatment without (A, B) and with (C, D) time as covariate. A B C D ROC curves of individual (A, C) GAM and their composites (B, D) (composite 1=nEla+MPO+NGAL+MMP-8; composite 2=nEla+MPO+NGAL+MMP-8+TIMP-1). For numerical values of AUC (95% CIs), specificity and sensitivity, see table 4. AUC, area under the curve; MMP-8, matrix metalloproteinase 8; MPO, myeloperoxidase; nEla, neutrophil elastase; NGAL, neutrophil gelatinase-associated lipocalin; NMOSD, neuromyelitis optica spectrum disorder; ROC, receiver operating characteristics; RRMS, relapsing-remitting multiple sclerosis; TIMP-1, tissue inhibitor of metalloproteinase-1.
to discriminate between NMOSD and RRMS further (not shown). When the risk of overfitting was addressed by calculating the AUC on 500 bootstrap replicates, these results were confirmed. Accordingly, the optimism-corrected AUCs showed a minimal reduction of both composite 1 and 2 to discriminate between NMOSD and RRMS (table 4). In essence, in untreated patients with NMOSD, with inclusion of time since disease exacerbation, specificity and sensitivity scores of these composites were within the same range as gold-standard live cell-based detection platforms for aAQP4, and better than referring ELISA-based assays27 (table 5). In patients with aAQP4− NMOSD, 71% overall (5/7 with and without corticosteroid or immunomodulatory pretreatment) and 100% (4/4) without corticosteroid pretreatment have been diagnosed based on GAM composite models as NMOSD; the two patients with corticosteroid or immunomodulatory pretreatment) and 100% (4/4) without corticosteroid pretreatment have been diagnosed based on GAM composite models as NMOSD; the two patients with corticosteroid pretreatment were classified as RRMS (table 6). On the group level, current results suggest that such therapy has only limited capacity to reduce GAM expression in the course of NMOSD exacerbation. Most other markers tested here displayed overlapping concentration ranges in acute stages of NMOSD versus RRMS, making them unsuitable for differentiating the two diseases in case of individual exacerbations. Furthermore, their levels did not correlate with disability scores, likely because their modulation reflects downstream effects in the course of the disease.

**DISCUSSION**

Current results demonstrate that GAM produce a humoral footprint in CSF that can be used clinically to differentiate these two diseases with equal sensitivity and specificity as aAQP4 in a setting of first disease exacerbation. Our findings also establish GAM as disease activity marker by the correlation of their levels with clinical severity at NMOSD exacerbation, a feature that distinguishes them from the purely diagnostic capacity of aAQP4. Moreover, as GAM are also upregulated in aAQP4− NMOSD, they can close a diagnostic gap for these patients. Accordingly, metabolomic approaches have allowed to differentiate with high accuracy aAQP4− NMOSD versus MS based on increased plasma levels of myoinositol and formate in the latter disease; different from our study cohort these results were derived from an out of relapse population and it is not known whether the differentiation between the two disease would apply as well in acute disease.

The correlations of GAM levels with CSF granulocytosis and acute disability scores strengthen the concept of a pathogenetic link between recruitment and activation of granulocytes, neural tissue damage and development of disability in NMOSD. In this context, it is notable that a significant number of patients with acute and s/c NMOSD had markedly increased GAM concentrations, despite corticosteroid or immunomodulating therapy prior to sampling. On the group level, current results suggest that such therapy has only limited capacity to reduce GAM expression in the course of NMOSD exacerbation.

---

**Table 5** Comparison of validity measures of biomarker composites and aAQP4 testing to differentiate between acute NMOSD and acute RRMS

<table>
<thead>
<tr>
<th>Time included in ROC model</th>
<th>N</th>
<th>AUC†</th>
<th>Sensitivity†</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Composite 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nEla, MPO, NGAL, MMP-8</td>
<td>No</td>
<td>57</td>
<td>90 (79 to 100)</td>
<td>87 (62 to 96)†</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>55</td>
<td>96 (90 to 100)</td>
<td>100 (77 to 100)†</td>
</tr>
<tr>
<td><strong>Composite 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nEla, MPO, NGAL, MMP-8, TIMP-1</td>
<td>No</td>
<td>57</td>
<td>94 (84 to 100)</td>
<td>87 (62 to 96)†</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>55</td>
<td>98 (94 to 100)</td>
<td>92 (67 to 99)†</td>
</tr>
<tr>
<td>aAQP4 test performance*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-IIF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EI-M1/M23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fixed cell-based assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live cell-based assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Pain et al.† Values are 95% CI.
†Sensitivity values were calculated by Wilson method.
‡Data based on modelling of combined cohorts, with patients without corticosteroid pretreatment.

**Table 6** Identification of patients with aAQP4− NMOSD by GAM composite algorithms

<table>
<thead>
<tr>
<th>Disease stage</th>
<th>Cohort</th>
<th>Pretreatment</th>
<th>Corticosteroids</th>
<th>Immunomodulators</th>
<th>Detected by composite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute</td>
<td>Discovery</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>s/c</td>
<td>Discovery</td>
<td>Intraoral −4 days of lp+oral</td>
<td>No</td>
<td>Azathioprine</td>
<td>Yes</td>
</tr>
<tr>
<td>s/c</td>
<td>Discovery</td>
<td>Oral</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Acute</td>
<td>Validation</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Acute</td>
<td>Validation</td>
<td>Intraoral −40 days of lp</td>
<td>IVIG −8 days of lp</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>s/c</td>
<td>Validation</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>s/c</td>
<td>Validation</td>
<td>(intraoral/tapering −11 weeks of lp)†</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Based on models without time factor.
†This patient was not counted as ‘corticosteroid pretreated’, because the biological activity of the drug is unlikely to be present anymore.

aAQP4, anti-aquaporin-4 antibody; AUC, area under the curve; EI-IIF, tissue-based indirect immunofluorescence; TIMP-1, tissue inhibitor of metalloproteinase-1.
inflammatory response in NMOSD. Nevertheless, generic tissue
injury-markers, such as NL and GFAP may still be clinically
useful, as they allow to monitor disease activity and therapeutic
response in NMOSD and may predict its long-term disability
course, not the least since blood-based samples allow for longitudi-
nal assessments. 6,29 30 Despite not being granulocyte products,
the leucocyte adhesion molecules VCAM-1 and ICAM-1 were
increased in NMOSD compared with RRMS in present results
and as found by others. 12 Their increase may be an indirect result
of the release of enzymes in the course of granulocyte activation,
since both molecules are substrates for proteolysis by elastase
and other neutrophil secretory enzymes. 25 26

The half-life time and kinetics of GAM under physiological
conditions and in disease are unknown and may show incon-
gruent kinetics among them. Accordingly, as the time between
start of their release in the course of disease exacerbation and
lumbar puncture may vary, the individual levels of GAM did not
show a consistent pattern of correlation. These findings have
their correlate in a recent study in patients with type 2 diabetes
mellitus where increased serum levels of nEla and MPO showed
only a moderate (r=0.56) correlation. 31 Hence, the rational
for the use of GAM composites, rather than a single marker, for
the differentiation between NMOSD and MS is to compensate
the variability of their levels at respective times of lumbar punc-
ture. An advantage of the proposed GAM composites is that they
rest on an analytical platform, ELISA, that is simple to execute
and technically robust and allows to differentiate patients with
aAQP4-IgG versus NMOSD from MS, this within a day of sampling as
compared with a 1–2 weeks laboratory turnaround time of gold-
standard cell-based assays for aAQP4. 32 33 Both aspects are clinically
important, as the diagnosis of acute NMOSD necessitates
a seamless start of plasma exchange (PEX), to optimise its effec-
tiveness. 34–36 PEX may not remain the only therapeutic option as
novel immunomodulatory therapies specifically interfering with
effector molecules of NMOSD pathogenesis, such as protease
and complement inhibitors, may emerge as acute phase therapies.
For example, eculizumab is currently registered only as interval
therapy for secondary prophylaxis against acute exacerbations
of NMOSD. However, this compound is in off-label use in acute
phases of haemolytic-uraemic syndrome 17 among other diseases
that go along with acute complement factor 5 (C5) activation
and may also be a therapeutic option in patients with NMOSD
with acute exacerbations when PEX provides only limited or no
benefit. 38 Here, GAM composites may be a valuable biomarker
for therapeutic decision making, on the background of the enor-
mous costs of anti-CS-antibody therapies.

The clinical finding of a correlation of GAM with neurological
impairment corroborates a large body of evidence for the
pathogenic role of granulocytes and their secretory products
in preclinical models of NMOSD. Thus, granulocyte depletion
preserves blood-brain barrier integrity and reduces lesional
damage in in vivo rodent models of NMOSD, while induction of
a neutrophilic state by granulocyte colony-stimulating factor led
to increased neural damage. 6 15 An ex vivo model of NMOSD
showed extensive potentiation of complement-mediated spinal
cord damage by the addition of elastase, 16 which could partly be
suppressed by the elastase-inhibitor sivelstat and other inhibi-
tors of neutrophil enzymes. 16 39 40 Sivelestat also demonstrated
therapeutic effects in a rodent in vivo model of MOGAD, but not
in MS-like experimental autoimmune encephalomyelitis. 41 This
study also observed increased serum levels of nEla in patients with
NMOSD and provided a possible explanation why interferon-β
seems to induce NMOSD exacerbations in humans, since this
cytokine induces the release of nEla in cultured granulocytes. 41

Limitations
The diagnostic capacity of GAM was only evaluated in NMOSD
versus RRMS, while increasing evidence suggests that granulo-
cytes are also involved in the pathogenesis of MOGAD, that is
as well difficult to distinguish in acute stage from RRMS and
NMOSD. 18 19 In a preliminary report, we have found that patients
with MOGAD, similarly to NMOSD, displayed a GAM pattern that
differentiated it from RRMS. 42 We are currently extending these preliminary data based on a larger cohort of
patients with MOGAD, in an attempt to explore possible qualita-
tive and quantitative differences of biomarker profiles between
this condition, RRMS and NMOSD. Second, there is a need to
expand the database of the capacity of GAM to identify aAQP4-
NMOSD, as the number of patients is currently small.

CONCLUSIONS
Current findings establish GAM as first biofluid markers of
NMOSD reflective of the clinical degree of neurological impair-
ment. Second, they establish GAM as an alternative biomarker to
aAQP4 for the differential diagnosis of NMOSD versus
RRMS, also comprising aAQP4 disease that shares with typical
NMOSD granulocyte activation as a common pathomechanism.
Third, together with previous preclinical evidence that inhibition
of proteolytic activity of granulocyte-derived enzymes inhibits
tissue damage in NMOSD models, this study identifies GAM as
potential novel drug targets for acute-stage NMOSD.

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and JK. Supervision: DL, MW, FP and JK.

Funding This investigation was supported by Swiss National Science Foundation
(grant 320030_1891401), the Health and Labour Sciences Research Grant on
Intractable Diseases (Neuroimmunological Diseases) from the Ministry of Health,
Labour and Welfare of Japan (20FC1030) and Swedish MRC grant no. 2020-02700,
Hjärnfonden.

Competing interests DL is Chief Medical Officer of GenoNeuro. MW received
speaker honoraria from Novartis Pharma, Chugai Pharmaceutical, Biogen Japan
and Alexion. FP has received research grants from Janssen, Merck KGaA and UCBI,
and fees for serving on DMC in clinical trials with Chugai, Lundbeck and Roche,
and preparation of witness report for Novartis. RF has received speaker fees for
teaching and workshops from Biogen, Merck, Novartis, Roche, Teva and Alexion.
For educational activities, courses or research, he has received unrestricted grants from
Biogen, EMD Serono. JL is an employee of Quanterix. BE has received travel grants for
ECTRIMS 2018 from Roche. KF has served on advisory boards and received
Neuro-inflammation

J Neurol Neurosurg Psychiatry: first published as 10.1136/jnnp-2022-330796 on 19 April 2023. Downloaded from http://jnnp.bmj.com/ on June 23, 2023 by guest. Protected by copyright.

J Neurol Neurosurg Psychiatry 2023;9:e11486:1–11.


