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# 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 pathoanatomical 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, matrixmetalloproteinase-8, tissue inhibitor of metalloproteinase-1), as well as a set of inflammatory and tissue-destruction markers, known to be upregulated in NMOSD and MS (neurofilament 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  $\leq 21$  days from onset of clinical exacerbation. Composites of GAM provided area under the curve values of 0.90–0.98 (specificity of 0.76–1.0, sensitivity of 0.87–1.0) to differentiate NMOSD from MS, including all anti-aquaporin-4 protein (aQP4)-antibody-negative patients who were untreated.

**Conclusions** GAM composites represent a novel biomarker to reliably differentiate NMOSD from MS, including in aQP4<sup>-</sup> 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.

## INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD) and relapsing-remitting multiple sclerosis (RRMS)

## 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 (aQP4).
- ⇒ 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 aQP4 in acute stages of disease, including also patients with aQP4<sup>-</sup> 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.

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.<sup>1</sup> The detection of auto-antibodies targeting the astrocyte 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<sup>-</sup> which makes it even more difficult to establish the accurate diagnosis.<sup>2–5</sup> Furthermore, the presence or the titre of aAQP4 is not related to clinical disease characteristics.<sup>4</sup> 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),<sup>6 7</sup> neurofilament light chain (NfL, a marker of neuroaxonal injury),<sup>6 8</sup> chemokine (C-X-C motif) ligand 13 (CXCL13, a B-cell attractant),<sup>9–11</sup> intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) (both leucocyte adhesion molecules)<sup>12</sup> and matrix metalloproteinase-9 (MMP-9, a matrix remodelling gelatinase).<sup>13</sup> 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.<sup>6</sup>

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).<sup>14–20</sup> 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<sup>-</sup> 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.<sup>16</sup> 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.<sup>21 22</sup> Acute disease exacerbation/relapse was defined according to 2017 McDonald criteria.<sup>22</sup> Disability was assessed using the Expanded Disability Status Scale (EDSS).<sup>23</sup> 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

	Cellular source in CSF		Function/Biomarker significance	Analytical platform	LOD (pg/mL)
Neutrophil elastase (EC 3.4.21.37)*	Granulocytes	Primary granule	Proteolytic enzyme	EnzChek Elastase Assay Kit (ThermoFisher)	18.2
Myeloperoxidase (EC 1.11.2.2)*				Hypochlorous acid (HOCl) production	SP-X platform (Simoa) (Quanterix)
Matrix metalloproteinase-8 (MMP-8, neutrophil collagenase, EC 3.4.24.34*)		Secondary granule	MMPs: extracellular matrix modulation		0.65
Neutrophil gelatinase-associated lipocalin†			NGAL: bacteriostatic by Fe chelation		0.33
Matrix metalloproteinase-9 (MMP-9, gelatinase B, EC 3.4.24.35)*		Tertiary granule	ubiquitous	Quantikine ELISA (R&D Systems)	50.6
Tissue inhibitor of metalloproteinase-1 (TIMP-1)‡		Secretory vesicles		SP-X platform (Simoa) (Quanterix)	0.74
S100 calcium-binding protein B	In CNS: astrocytes		Cytoplasmatic calcium-binding protein, cell damage marker	ELISA (BioVendor)	3.77
Glial fibrillary acidic protein			Cytoskeleton intermediate filament, cell damage marker	HD-X platform (Simoa) (Quanterix)	0.21
Neurofilament light chain	Neurons				0.04
Vascular cell adhesion protein-1 (CD106)	Endothelial cells		Adhesion molecules	SP-X platform (Simoa) (Quanterix)	1.12
Intercellular adhesion molecule-The supplemental materiaö1 (CD54)					0.42
C-X-C motif chemokine 13	In CNS: macrophage-like cells		B-cell chemoattractant		0.05

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.<sup>43</sup>

CNS, central nervous system; CSF, cerebrospinal fluid; LOD, limit of detection; Simoa, single molecular array.

**Table 2** Demographic and clinical characteristics of patient groups and control persons

		Discovery cohort			Validation cohort			Controls	
		NMOSD	RRMS	P value	NMOSD	RRMS	P value	INDC	SC
Patients male (n, %)		34 5 (14.7)	36 8 (22.2)	0.617	25 2 (8.0)	46 14 (30.4)	0.062	15 11 (73)	25 10 (40)
Samples (n) acute: s/c		42* 20:22	40* 17:23		25 21:4	46 34:12		15 –	25 –
Age at first CSF sample (years) mean (SD)		44.8 (11.7)	37.4 (12.7)	0.015	47.5 (18.0)	36.2 (11.4)	0.004	54.7 (19.9)	45.9 (9)
Disease duration at sampling median (IQR)		6.7 (2.0, 12.4)	6.0 (2.0, 11.0)	0.784	3.6 (0.4, 8.5)	1.9 (0.1, 9.2)	0.338	n/a	n/a
Interval between clinical attack and lumbar puncture† (days) median (IQR)		30.0 (8.0, 83.0)	40.5 (9.8, 93.8)	0.511	9.5 (7.0, 13.8)	13.5 (8.0, 20.8)	0.057	n/a	n/a
EDSS score median (IQR)		5.5 (3.0, 7.0)	3.5 (2.5, 6.0)	0.015	4.5 (3.5, 6.5)	3.0 (2.0, 3.5)	<0.001	n/a	n/a
aAQP4 <sup>-</sup> , n (%)		3‡ (8.8)	n/a	n/a	4 (16.0)	n/a	n/a	n/a	n/a
CSF samples with granulocytosis, (n/N, %)		9/42 (21.4)	2/40 (5.0)	0.063	5/14§ (35.7)	6/18 (33.3)	1.0	8 (53.3)	0
CSF cell count (×/μL) median (IQR)		3.0 (1.0, 5.8)	3.0 (1.0, 5.0)	0.985	2.5 (1.0, 11.8)	4.0 (1.5, 11.0)	0.561	37.7 (16.6, 102.3)	1.0 (0.6, 2.0)
Samples of patients with treatment prior lumbar puncture (n/N, %)	Corticosteroid	10 (23.8)	6 (15.0)	0.388	1 (4)	0 (0)	n/a	n/a	n/a
	Pulse intravenous¶								
	Oral	29 (69.0)	12 (33.3)	0.001	11 (44.0)	4 (8.7)	<0.001		
	Immunomodulatory	10** (23.8)	1 (4.0)	0.012	4** (16.0)	11 (23.9)	0.634	n/a	n/a

\*Six patients with NMOSD and four patients with RRMS contributed >1 CSF sample from independent disease exacerbations (see 'Methods' section).

†The time between disease exacerbation and lumbar puncture was significantly longer in the discovery versus validation cohorts (NMOSD+RRMS) (days, median (IQR)): 36.0 (10.0, 83.0) vs 11.5 (7.8, 18.2), p<0.001.

‡One patient had a lumbar puncture after start of corticosteroid therapy and scored aAQP4<sup>-</sup> by cell-based assay but became positive when tested during a later attack. Six of seven aAQP4<sup>-</sup> patients scored also negative for anti-MOG antibodies, one patient was not tested.

§In the validation set the CSF granulocyte cell count was not reported for 11 NMOSD and 28 RRMS samples.

¶The time range of administration prior lumbar puncture was 1–40 days; 7 patients with NMOSD in the discovery set had received intravenous corticosteroid and oral therapy; 1 and 2 patients in the discovery and validation set, respectively, had received intravenous corticosteroids 9–12 weeks before lumbar puncture and were not used for statistical calculations on corticosteroid effects. Three patients with RRMS in the discovery set had received intravenous corticosteroid and oral therapy.

\*\*In total, 14 patients with NMOSD were on immunomodulatory therapy (azathioprine: 7, tacrolimus: 4, methotrexate: 2, cyclophosphamide: 1) at the time point of lumbar puncture. In addition, 2 patients in the discovery set (patient 1: 45–47 and 1–5 days before lumbar puncture; patient 2: 23–27 days before lumbar puncture) and one in the validation cohort (patient 3: 8 days before lumbar puncture) had received intravenous immunoglobulins.

aAQP4, anti-aquaporin-4 antibody; EDSS, Expanded Disability Status Scale; INDC, inflammatory neurological disease control; n/a, not available; NMOSD, neuromyelitis optica spectrum disorder; RRMS, relapsing-remitting multiple sclerosis; SC, symptomatic control.

were used to determine values under physiological and highly inflammatory conditions, respectively: 'symptomatic controls' (SC)<sup>24</sup> consisted of 25 patients in whom a structural neurological disease was excluded, based on normal findings in clinical and 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<sup>24</sup> (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/myelomeningoradiculitis 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, as well as the interaction between these two variables, were used as independent variables. The interaction indicates whether the temporal dynamics differ between patients with

**Table 3** Levels of biomarkers, all patients

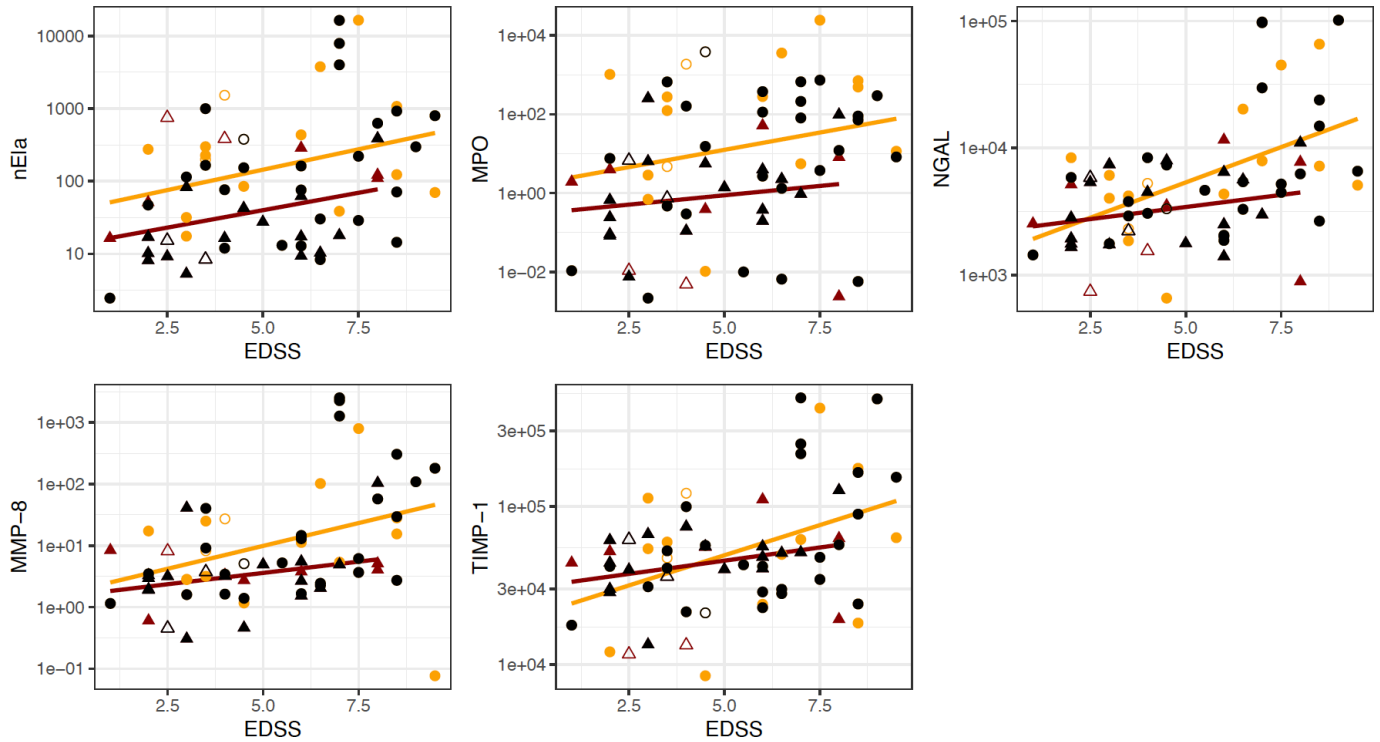
Disease group (n)	Discovery				Validation											
	NMOSD All (42)*	RRMS All (40)*	P value	NMOSD Acute (20)	NMOSD s/c (22)	P value	RRMS Acute (17)	P value†	NMOSD All (25)	RRMS All (46)	P value	NMOSD Acute (21)	NMOSD s/c (4)	P value	RRMS Acute (34)	P value†
nEla	42.0 (15.6, 162.6)	15.3 (6.5, 43.4)	0.001	114.8 (44.9, 670.8)	17.1 (10.2, 42.3)	<0.001	15.2 (8.3, 38.9)	<0.001	220.7 (83.7, 435.2)	26.1 (13.8, 139.3)	0.003	220.7 (14.4, 435.2)	253.3 (112.3, 476.4)	0.858	17.2 (9.6, 100.2)	0.008
MPO	3.8 (0.7, 10.7)	1.2 (0.4, 3.0)	0.013	7.9 (2.8, 73.7)	1.2 (0.3, 5.2)	0.003	0.6 (0.2, 3.4)	0.001	253.7 (706.8)	15.1 (15.1, 161.2)	0.016	280.9 (0.0, 731.7)	0.0 (0.0, 63.4)	0.068	33.5 (0.0, 165.8)	0.005
NGAL	5528 (2846, 7877)	3809 (2113, 5137)	0.001	6411 (5173, 17148)	4012 (2292, 6302)	0.010	2210 (2087, 4374)	<0.001	3289 (1762, 4642)	1526 (1130, 2349)	<0.001	3786 (2054, 5263)	1216 (849.4, 1594)	0.004	1506 (1101, 2570)	<0.001
MMP-8	3.6 (2.5, 9.0)	2.5 (0.7, 3.8)	0.004	8.7 (3.3, 127.3)	3.1 (1.9, 4.6)	0.005	2.6 (1.8, 2.7)	0.001	6.2 (2.7, 25.1)	1.4 (1.1, 3.1)	<0.001	6.2 (2.2, 25.1)	6.1 (3.9, 16.4)	0.803	1.4 (1.1, 3.1)	<0.001
MMP-9	136.1 (23.9, 1923)	101.6 (25.4, 494.6)	0.400	270.2 (37.3, 2195)	36.9 (18.6, 1660)	0.265	174.3 (26.5, 1031)	0.577								
TIMP-1	55.9 (44.1, 85.7)	39.2 (35.6, 43.4)	<0.001	62.7 (51.2, 155.8)	51.6 (41.4, 61.6)	0.032	38.0 (34.3, 39.6)	<0.001	24.2 (18.3, 42.8)	12.6 (10.7, 18.1)	<0.001	28.8 (21.6, 47.7)	13.4 (12.9, 14.9)	0.011	13.3 (10.9, 18.3)	<0.001
GFAP	14.6 (8.1, 39.3)	7.5 (5.3, 9.3)	<0.001	34.0 (10.7, 611.2)	12.2 (7.4, 19.4)	0.060	7485 (5541, 11321)	0.001								
S100B	219.6 (166.5, 311.9)	175.8 (135.6, 202.8)	0.009	249.2 (186.0, 496.6)	199.7 (155.4, 246.6)	0.030	168.3 (153.1, 188.3)	0.005	291.8 (192.4, 448.6)	184.8 (147.3, 242.3)	0.003	295.3 (192.4, 448.6)	199.1 (161.8, 528.3)	0.592	184.8 (150.4, 260.1)	0.006
NfL	1739 (1168, 5156)	1980 (1249, 4391)	0.981	1732 (1135, 3697)	2653 (1254, 6129)	0.371	2436 (1397, 8061)	0.493								
ICAM-1	3198 (1968, 4196)	2008 (1513, 2474)	<0.001	3698 (2005, 6225)	2562 (1880, 3837)	0.116	1956 (1720, 2560)	0.002	1729 (1300, 2528)	1286 (957.6, 1693)	0.011	2301 (1489, 2612)	1037 (982.4, 1146)	0.020	1335 (988.7, 1693)	0.003
VCAM-1	252.2 (202.4, 362.4)	209.3 (161.6, 238.2)	0.001	266.9 (226.2, 464.2)	230.0 (184.4, 289.5)	0.087	219.8 (173.5, 245.2)	0.010	160.4 (132.5, 267.5)	107.1 (81.5, 172.9)	0.008	167.5 (140.6, 281.1)	97.4 (88.5, 116.3)	0.045	128.5 (91.0, 172.9)	0.006
CXCL13	7.4 (2.5, 41.2)	4.8 (2.0, 21.3)	0.228	11.4 (3.9, 47.9)	3.4 (2.0, 29.2)	0.149	6.7 (2.9, 21.5)	0.326								

Values are medians (QR) in pg/mL for nEla, MPO, NGAL, MMP-8, MMP-9, S100B, NfL and ICAM-1, and in pg/mL × 10<sup>3</sup> 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 with acute RRMS.

CSF, cerebrospinal fluid; CXCL13, C-X-C motif chemokine 13; GFAP, glial fibrillar acidic protein; ICAM-1, intercellular adhesion molecule-1; MMP, matrix metalloproteinase; MPO, myeloperoxidase; N, number of sample; nEla, neutrophil elastase; NfL, neurofilament; NGAL, neutrophil gelatinase-associated lipocalin; NMOSD, neuromyelitis optica spectrum disorder; RRMS, relapsing-remitting multiple sclerosis; S100B, S100 calcium-binding protein B; s/c, subacute/chronic; TIMP-1, tissue inhibitor of metalloproteinase-1; VCAM-1, vascular cell adhesion molecule 1.



**Figure 1** Association between clinical disease severity and granulocyte activation markers levels in patients with NMOSD. Biomarker values are in pg/mL. Values on x-axis show the EDSS score at the time point of lumbar puncture. Regression lines show correlations in NMOSD patients without (acute ●; s/c: ▲) corticosteroid pre-treatment; patients with (acute: ●; s/c: ▲) corticosteroid pre-treatment. Open symbols designate aAQP4<sup>-</sup> patients. The Spearman’s correlation analysis of all, acute and s/c cohorts of patients showed significant correlations with the EDSS score; note that intercellular adhesion molecule-1 and vascular cellular adhesion molecule-1 levels correlated as well with EDSS scores (see online supplemental table 3). EDSS, Expanded Disability Status Scale; MMP-8, matrix metalloproteinase 8; MPO, myeloperoxidase; nEla, neutrophil elastase; NGAL, neutrophil gelatinase-associated lipocalin; NMOSD, neuromyelitis optica spectrum disorder; TIMP-1, tissue inhibitor of metalloproteinase-1.

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 ( $\leq 21$  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  $p=0.05$ .

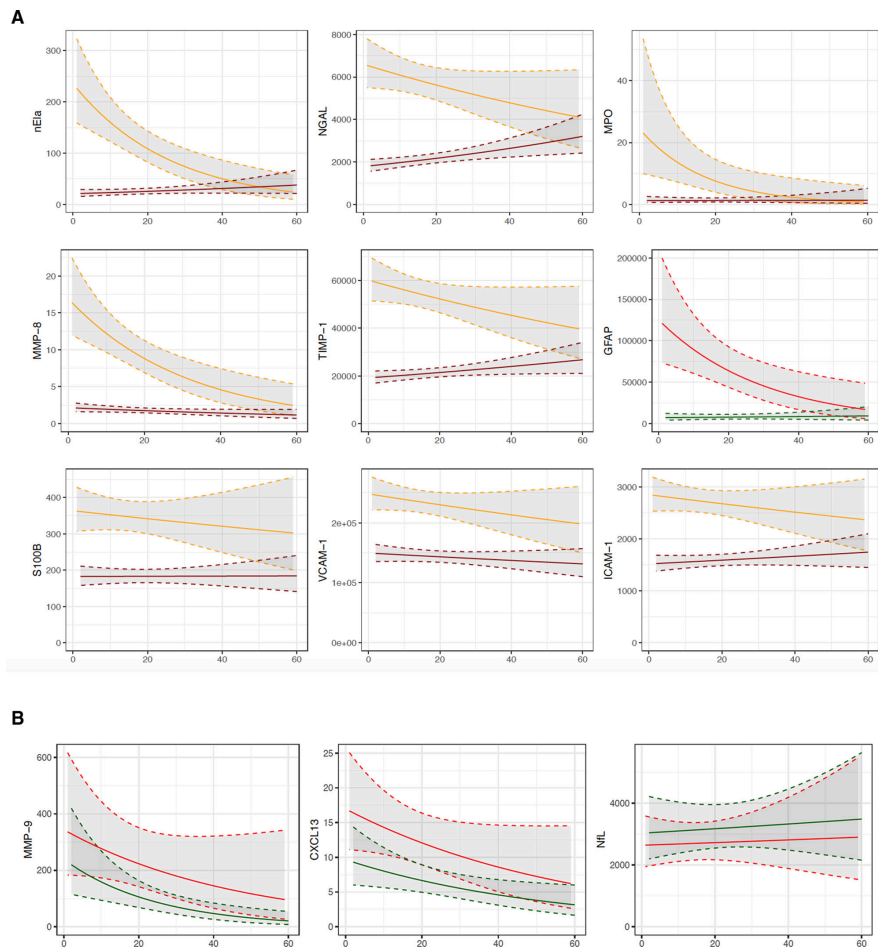
**RESULTS**

**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’ ( $\leq 21$  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<sup>+</sup>, 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



**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.

contrast, levels of NfL, MMP-9 and CXCL13 were not different for these three comparisons (table 3, online supplemental figure 1A).

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 INCD, while for MMP-9 levels were higher in INCD. 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 INCD (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

**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

Marker	Time	AUC		Youden Index	Specificity	Sensitivity	PPV	NPV
		Original (95% CI)	Optimism corrected					
nEla	–	0.85 (0.75 to 0.95)	0.81	0.14	0.64	0.93	0.48	0.96
	+	0.86 (0.72 to 0.99)	0.72	0.11	0.79	0.85	0.55	0.94
MPO	–	0.78 (0.65 to 0.91)	0.71	0.24	0.93	0.53	0.73	0.85
	+	0.74 (0.58 to 0.91)	0.59	0.21	0.81	0.62	0.50	0.87
NGAL	–	0.85 (0.71 to 0.99)	0.79	0.29	0.83	0.80	0.63	0.92
	+	0.91 (0.83 to 0.99)	0.84	0.21	0.79	0.92	0.57	0.97
MMP-8	–	0.81 (0.66 to 0.96)	0.73	0.16	0.71	0.87	0.52	0.94
	+	0.84 (0.71 to 0.96)	0.69	0.24	0.86	0.69	0.60	0.90
TIMP-1	–	0.82 (0.66 to 0.98)	0.77	0.33	0.93	0.73	0.79	0.91
	+	0.82 (0.65 to 0.98)	0.70	0.42	0.98	0.69	0.90	0.91
S100B	–	0.69 (0.49 to 0.89)	0.61	0.35	0.95	0.53	0.80	0.85
	+	0.81 (0.67 to 0.94)	0.70	0.34	0.98	0.54	0.88	0.87
ICAM-1	–	0.69 (0.51 to 0.88)	0.62	0.42	0.98	0.47	0.88	0.84
	+	0.76 (0.60 to 0.92)	0.63	0.34	0.95	0.46	0.75	0.85
VCAM-1	–	0.71 (0.54 to 0.89)	0.66	0.34	0.86	0.53	0.57	0.84
	+	0.74 (0.58 to 0.90)	0.63	0.28	0.79	0.62	0.47	0.87
nEla, NGAL, MPO, MMP-8; composite 1	–	0.90 (0.79 to 1.00)	0.84	0.22	0.81	0.87	0.62	0.94
	+	0.96 (0.90 to 1.00)	0.75	0.08	0.76	1.00	0.57	1.00
nEla, NGAL, MPO, MMP-8, TIMP-1; composite 2	–	0.94 (0.84 to 1.00)	0.89	0.52	1.00	0.87	1.00	0.95
	+	0.98 (0.94 to 1.00)	0.79	0.48	1.00	0.92	1.00	0.98

ROC curves are calculated on parameters estimated in the discovery cohort based on a logistic model. Youden Index as estimated in the pooled data cohort. The corrected AUC was calculated with 500 bootstrap runs.

AUC, area under the curve; MMP, matrix metalloproteinase; MPO, myeloperoxidase; nEla, neutrophil elastase; NGAL, neutrophil gelatinase-associated lipocalin; NMOSD, neuromyelitis optica spectrum disorder; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic; RRMS, relapsing-remitting multiple sclerosis; S100B, S100 calcium-binding protein; TIMP-1, tissue inhibitor of metalloproteinase-1.

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).

### Association between biomarker levels disease severity/disability status, aAQP4 status and CSF granulocyte count

Figure 1 shows that CSF levels of GAM in all (with or without therapy) patients with NMOSD were correlated with EDSS scores ( $\rho=0.31-0.46$ , all  $p\leq 0.01$ ). In acute NMOSD, this was also the case for NGAL, MMP-8 and TIMP-1 ( $\rho=0.39-0.50$ ,  $p<0.001-0.011$ , but not for nEla and MPO, while in s/c NMOSD only nEla was correlated with the EDSS score ( $\rho=0.41$ ,  $p=0.036$ ) (online supplemental table 3). GFAP levels, only analysed in the discovery set, did not correlate with the EDSS score in acute NMOSD and RRMS, while this was the case in s/c phase for NMOSD ( $\rho=0.58$  (0.21, 0.81),  $p=0.004$ ), and a referring trend was found for RRMS ( $\rho=0.40$  (–0.01, 0.81),  $p=0.004$ ). In the seven patients with aAQP4<sup>–</sup> NMOSD, GAM levels were similar to those of patients with aAQP4<sup>+</sup> NMOSD (figure 1). Interestingly, there was not a general downregulation of GAM levels across all corticosteroid-treated patients, but instead a random distribution with many patients scoring 1–2 logs above average GAM levels (figure 1). In contrast, S100B, NfL, MMP-9 and CXCL13 were not associated with EDSS scores or had  $\rho$  values  $\leq 0.29$  in NMOSD, with or without corticosteroid pretreatment; furthermore, in RRMS all these biomarkers showed only weak correlation ( $\rho\leq 0.3$ ) with EDSS scores (not shown).

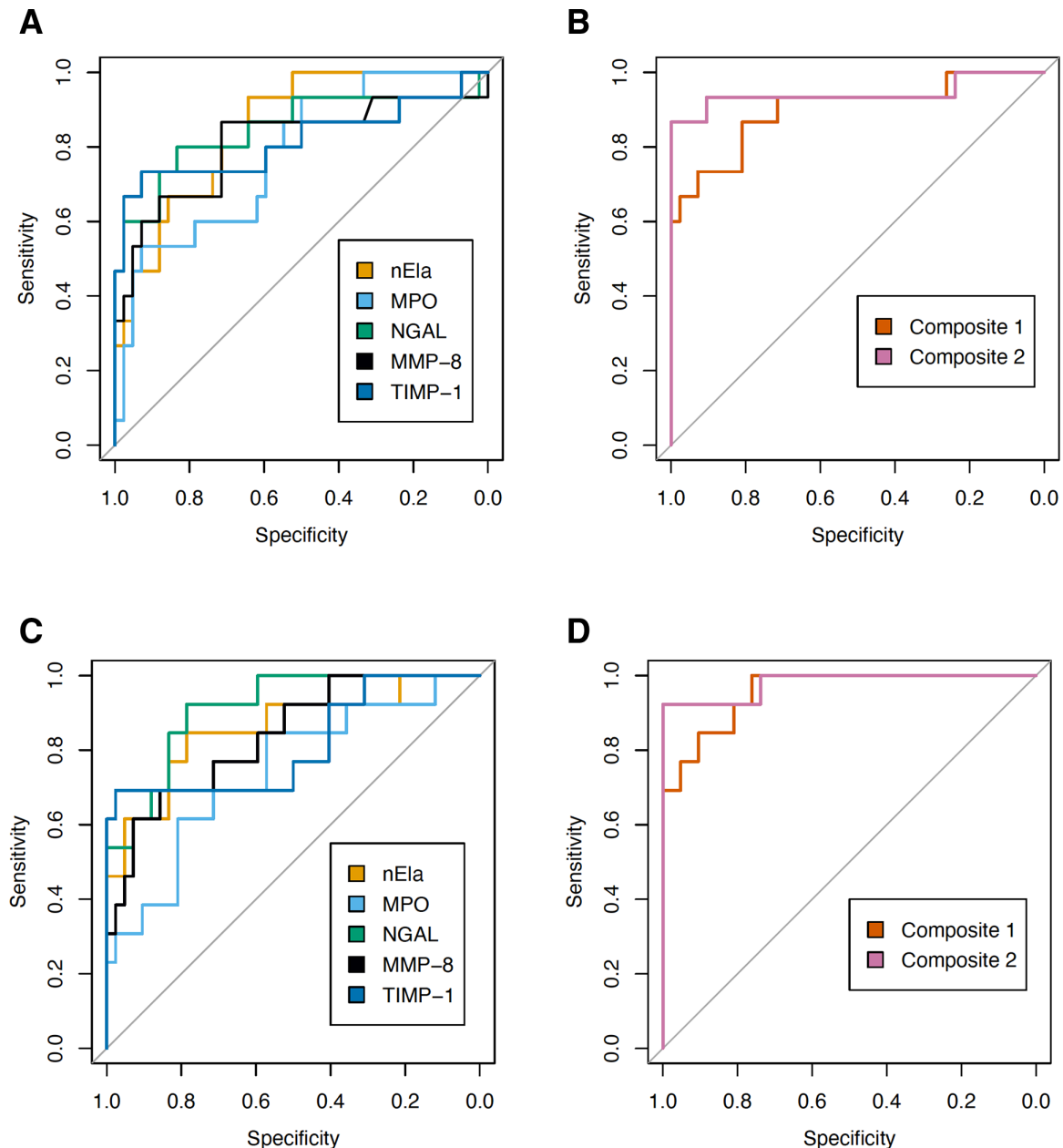
The adhesion molecules ICAM-1 and VCAM-1 were similarly associated with EDSS scores as GAM in all, and partly in

acute, NMOSD (online supplemental table 3). Being substrates for proteolytic cleavage from the cell surface by nEla and other granulocyte proteases,<sup>25,26</sup> 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.

### 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,  $\leq 21$  days after disease exacerbations. In contrast, MMP-9, CXCL13 and NfL



**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.

did not differ between acute stages of NMOSD and RRMS; the former two decreasing from onset in both conditions (pattern 2) and the latter increasing over time in NMOSD and RRMS (pattern 3).

### 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



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

	Time included in ROC model	N	AUC†	Sensitivity†	Specificity
<b>Composite 1§</b> nEla, MPO, NGAL, MMP-8	No	57	90 (79 to 100)	87 (62 to 96)‡	81
	Yes	55	96 (90 to 100)	100 (77 to 100)‡	76
<b>Composite 2§</b> nEla, MPO, NGAL, MMP-8, TIMP-1	No	57	94 (84 to 100)	87 (62 to 96)‡	100
	Yes	55	98 (94 to 100)	92 (67 to 99)‡	100
aAQP4 test performance*	T-IIF			79 (69 to 87)	100
	ELISA			60 (44 to 73)	97
	EI-M1/M23			90 (78 to 96)	95
	Fixed cell-based assay			94 (82 to 98)	98
	Live cell-based assay			92 (78 to 97)	100

\*Prain *et al.*<sup>27</sup>  
†Values are 95% CI.  
‡Sensitivity values were calculated by Wilson method.  
§Data based on modelling of combined cohorts, with patients without corticosteroid pretreatment.  
aAQP4, anti-aquaporin-4 antibody; AUC, area under the curve; EI-M1/M23, Neuroimmune M1/M23 biochip slide; MMP, matrix metalloproteinase; MPO, myeloperoxidase; nEla, neutrophil elastase; NGAL, neutrophil gelatinase-associated lipocalin; NMOSD, neuromyelitis optica spectrum disorder; ROC, receiver operating characteristics; RRMS, relapsing-remitting multiple sclerosis; T-IIF, tissue-based indirect immunofluorescence; TIMP-1, tissue inhibitor of metalloproteinase-1.

**Table 6** Identification of patients with aAQP4<sup>-</sup> NMOSD by GAM composite algorithms

Disease stage	Cohort	Pretreatment		Detected by composite	
		Corticosteroids	Immunomodulators	1*	2*
Acute	Discovery	No	No	Yes	Yes
s/c	Discovery	Intravenous –4 days of lp+oral	No	No	No
s/c	Discovery	Oral	Azathioprine	Yes	Yes
Acute	Validation	No	No	Yes	Yes
Acute	Validation	Intravenous –40 days of lp	IVIg –8 days of lp	No	No
s/c	Validation	No	No	Yes	No
s/c	Validation	(intravenous/tapering –11 weeks of lp)†	No	Yes	Yes

\*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; GAM, granulocyte activation markers; IVIg, intravenous immunoglobulins; lp, lumbar puncture; NMOSD, neuromyelitis optica spectrum disorder; s/c, subacute/chronic.

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 assays<sup>27</sup> (table 5). In patients with aAQP4<sup>-</sup> 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 NMOSD who scored negative in both algorithms had received intravenous corticosteroids or intravenous immunoglobulins 4 and 8 days prior lumbar puncture, respectively (table 6).

## 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.<sup>4</sup> Moreover, as GAM are also upregulated in aAQP4<sup>-</sup> NMOSD, they can close a diagnostic gap for these patients.<sup>5</sup> Accordingly, metabolomic approaches have allowed to differentiate with high accuracy aAQP4<sup>-</sup> 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 diseases would apply as well in acute disease.<sup>28</sup>

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.

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

inflammatory response in NMOSD. Nevertheless, generic tissue injury-markers, such as NfL 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 longitudinal assessments.<sup>6 29 30</sup> 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.<sup>12</sup> 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.<sup>25 26</sup>

The half-life time and kinetics of GAM under physiological conditions and in disease are unknown and may show incongruent 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 ( $\rho=0.56$ ) correlation.<sup>31</sup> Hence, the rationale 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 puncture. 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<sup>-</sup> 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.<sup>32 33</sup> Both aspects are clinically important, as the diagnosis of acute NMOSD necessitates a seamless start of plasma exchange (PEX), to optimise its effectiveness.<sup>34–36</sup> 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<sup>37</sup> 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.<sup>38</sup> Here, GAM composites may be a valuable biomarker for therapeutic decision making, on the background of the enormous costs of anti-C5-antibody therapies.

The clinical finding of a correlation of GAM with neurological impairment corroborate 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.<sup>16 39</sup> An ex vivo model of NMOSD showed extensive potentiation of complement-mediated spinal cord damage by the addition of elastase,<sup>40</sup> which could partly be suppressed by the elastase-inhibitor sivelestat and other inhibitors of neutrophil enzymes.<sup>16 39 40</sup> Sivelestat also demonstrated therapeutic effects in a rodent in vivo model of NMOSD, but not in MS-like experimental autoimmune encephalomyelitis.<sup>41</sup> This study also observed increased serum levels of nEla in patients with NMOSD and provided a possible explanation why interferon- $\beta$  seems to induce NMOSD exacerbations in humans, since this cytokine induces the release of nEla in cultured granulocytes.<sup>41</sup>

## Limitations

The diagnostic capacity of GAM was only evaluated in NMOSD versus RRMS, while increasing evidence suggests that granulocytes are also involved in the pathogenesis of MOGAD, that is as well difficult to distinguish in acute stage from RRMS and NMOSD.<sup>18 19</sup> In a preliminary report, we have found that patients with MOGAD, similarly to NMOSD, displayed a GAM pattern that differentiated it from RRMS.<sup>42</sup> We are currently extending these preliminary data based on a larger cohort of patients with MOGAD, in an attempt to explore possible qualitative 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<sup>-</sup> 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 impairment. Second, they establish GAM as an alternative biomarker to aAQP4 for the differential diagnosis of NMOSD versus RRMS, also comprising aAQP4<sup>-</sup> 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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Original Investigation  
GAM\_NMOSD\_JNNP\_230223\_SupplMat\_edited030423\_track

Granulocyte markers identify acute NMOSD

**Supplemental Tables and Figures****Supplemental Table 1. Levels of biomarkers in acute NMOSD (discovery cohort) vs INDC and SC, discovery cohort**

Marker type	NMOSD acute	INDC	P	SC	P <sup>‡</sup>
Number of samples	20	15		25	
nEla	114.8 [44.9, 670.8]	94.8 [49.8, 255.7]	1.000	13.2 [9.2, 17.6]	<0.001
MPO	7.9 [2.8, 73.7]	6.1 [2.5, 55.7]	0.610	1.0 [0.2, 2.2]	<0.001
NGAL	6410.5 [5173.0, 17147.5]	9479.0 [5188.0, 15685.0]	0.499	3751.0 [2221.0, 5111.0]	<0.001
MMP-8	8.7 [3.3, 127.3]	2.7 [2.3, 6.4]	0.043	2.2 [1.6, 2.7]	<0.001
MMP-9	270.2 [37.3, 2195.0]	9850.5 [832.3, 51865.4]	0.003	30.8 [20.3, 40.1]	0.006
TIMP-1	62660.0 [51160.0, 155750.0]	82970.0 [54600.0, 131450.0]	0.633	37490.0 [35530.0, 44650.0]	<0.001
GFAP	33958.8 [10730.5, 611223.2]	12125.3 [9313.4, 25050.4]	0.131	9818.0 [6725.0, 11551.7]	0.002
S100B	249.2 [186.0, 496.6]	301.1 [222.8, 402.4]	0.755	248.0 [175.4, 287.0]	0.230
NfL	1732.0 [1134.8, 3696.9]	1278.3 [448.4, 5334.6]	0.188	436.8 [316.3, 517.2]	<0.001
ICAM-1	3697.5 [2004.5, 6224.5]	4862.0 [3323.0, 9052.0]	0.131	2066.0 [1744.0, 2441.0]	0.004
VCAM-1	266900.0 [226175.0, 464200.0]	353000.0 [275600.0, 449000.0]	0.400	157400.0 [133700.0, 209000.0]	<0.001
CXCL13	11.4 [3.9, 47.9]	8.5 [2.5, 84.9]	0.877	0.6 [0.5, 0.9]	<0.001

Biomarker values are medians [IQR] in pg/ml. P values  $\leq 0.05$  are with green background.<sup>‡</sup>P of comparison of acute NMOSD with SC.

Abbreviations: CXCL13: C-X-C motif chemokine 13; GFAP: glial fibrillary acidic protein; ICAM-1: intercellular adhesion molecule-1; INDC: inflammatory neurological disease control; 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; S100B: S100 calcium-binding protein B; SC: symptomatic control; TIMP-1: tissue inhibitor of metalloproteinase-1; VCAM-1: vascular cell adhesion molecule-1.

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Granulocyte markers identify acute NMOSD

**Supplemental Table 2. GAM levels in all NMOSD patients with and without corticosteroid pre-treatment**

Corticosteroid pre-treatment (N)	No 23	Yes 44	P
Days between disease exacerbation and lumbar puncture; median [IQR]	13.0 [6.0, 43.5]	13.0 [7.0, 44.2]	0.982
nEla	199.7 [60.6, 409.9]	36.4 [13.0, 179.4]	0.006
MPO	8.2 [1.3, 385.3]	3.8 [0.3, 91.6]	0.214
NGAL	5095.0 [2423.0, 7837.0]	4480.0 [2180.0, 6760.9]	0.870
MMP-8	5.4 [3.0, 16.4]	3.6 [2.0, 18.4]	0.499
TIMP-1	52020.0 [21725.0, 63212.0]	45870.0 [30832.5, 63017.5]	0.891
S100B	225.3 [159.9, 606.2]	225.9 [182.7, 309.0]	0.900
ICAM-1	3531.0 [1305.5, 4488.0]	2312.5 [1826.0, 3772.5]	0.932
VCAM-1	230900.0 [159450.0, 31685.0]	212000.0 [158250.0, 305425.0]	0.716

Biomarker values are medians [IQR] in pg/ml. P values  $\leq 0.05$  are with green background.

Abbreviations: ICAM-1: intercellular adhesion molecule-1; MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NGAL: neutrophil gelatinase-associated lipocalin; NMOSD: neuromyelitis optica spectrum disorder; S100B: S100 calcium-binding protein B; TIMP-1: tissue inhibitor of metalloproteinase-1; VCAM-1: vascular cell adhesion molecule-1.

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Granulocyte markers identify acute NMOSD

**Supplemental Table 3. Association between EDSS and biomarkers in samples of NMOSD patients with and without corticosteroid pre-treatment**

Marker	all NMOSD N=67		acute NMOSD N=41		s/c NMOSD N=26	
	Rho [CI]	p	Rho [CI]	p	Rho [CI]	p
nEla	0.38 [0.16-0.57]	0.001	0.24 [-0.11-0.48]	0.135	0.41 [0.03-0.69]	0.036
MPO	0.31 [0.08-0.52]	0.010	0.20 [-0.1-0.48]	0.213	0.21 [-0.19-0.56]	0.293
NGAL	0.46 [0.24-0.63]	<0.001	0.50 [0.22-0.70]	<0.001	0.27 [-0.13-0.60]	0.181
MMP-8	0.40 [0.18-0.59]	<0.001	0.40 [0.11-0.63]	0.009	0.27 [-0.13-0.60]	0.178
TIMP-1	0.35 [0.12-0.54]	0.004	0.39 [0.10-0.63]	0.011	0.28 [-0.12-0.60]	0.168
ICAM-1	0.39 [0.16-0.57]	0.002	0.47 [0.19-0.68]	0.002	0.18 [-0.22-0.53]	0.375
VCAM-1	0.38 [0.15-0.57]	0.001	0.46 [0.17-0.67]	0.003	0.14 [-0.26-0.50]	0.501

P values  $\leq 0.05$  are with green background.

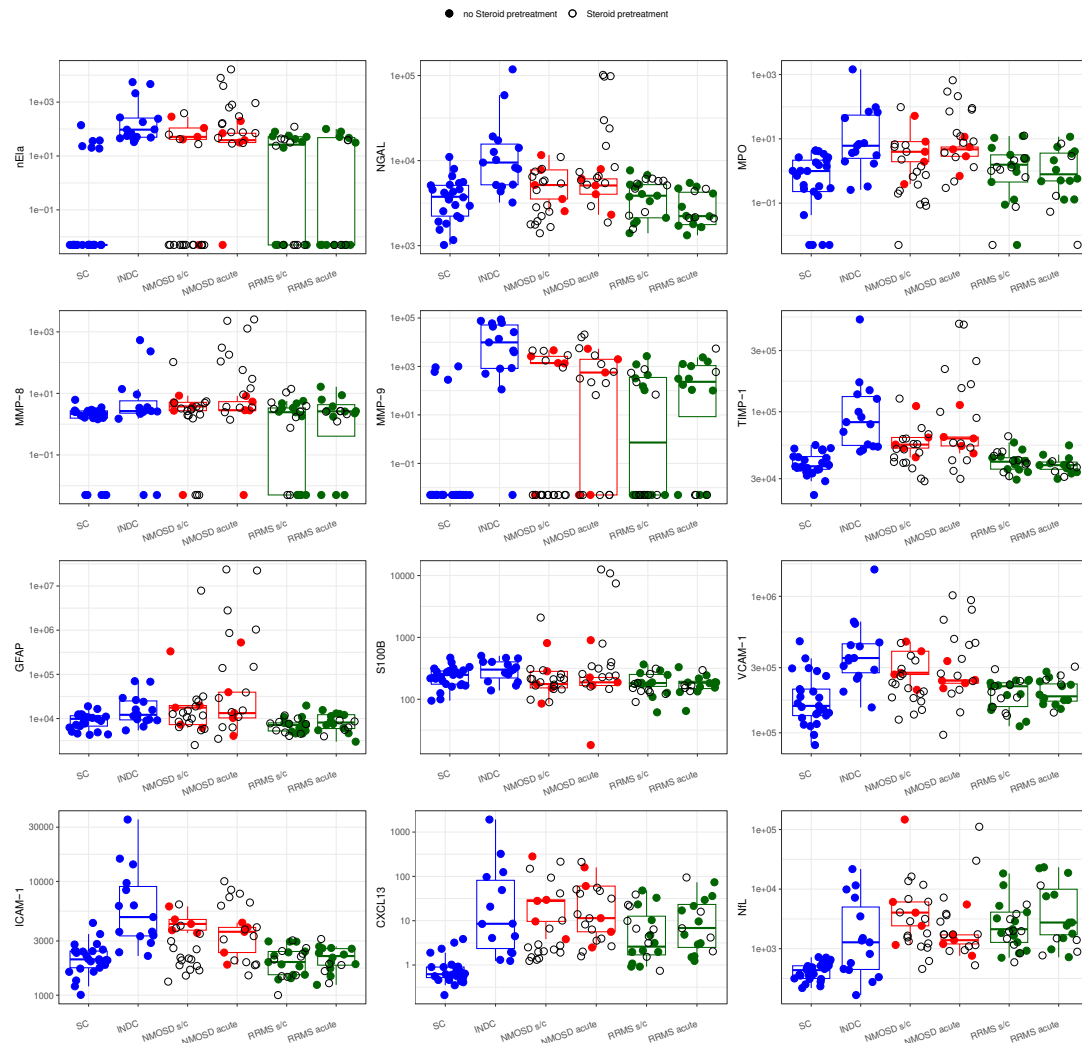
Abbreviations: CXCL13: C-X-C motif chemokine 13; GFAP: glial fibrillary acidic protein; ICAM-1: intercellular adhesion molecule-1; MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NGAL: neutrophil gelatinase-associated lipocalin; NfL: neurofilament light chain; NMOSD: neuromyelitis optica spectrum disorder; RRMS: relapsing-remitting multiple sclerosis; TIMP-1: tissue inhibitor of metalloproteinase-1; S100B: S100 calcium-binding protein B; VCAM-1: vascular cell adhesion molecule-1.

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### Supplemental Figure 1A. Biomarker levels in NMOsD, RRMS, SC and INDCs in discovery cohort



Boxplot of biomarker levels on log-scale (pg/ml) by patient population. Patients with corticosteroid pre-treatment (○) are not used for the boxplot calculations. Note that many patients under corticosteroid treatment in acute NMOsD have values above the 3<sup>rd</sup> quartile/75<sup>th</sup> percentile.

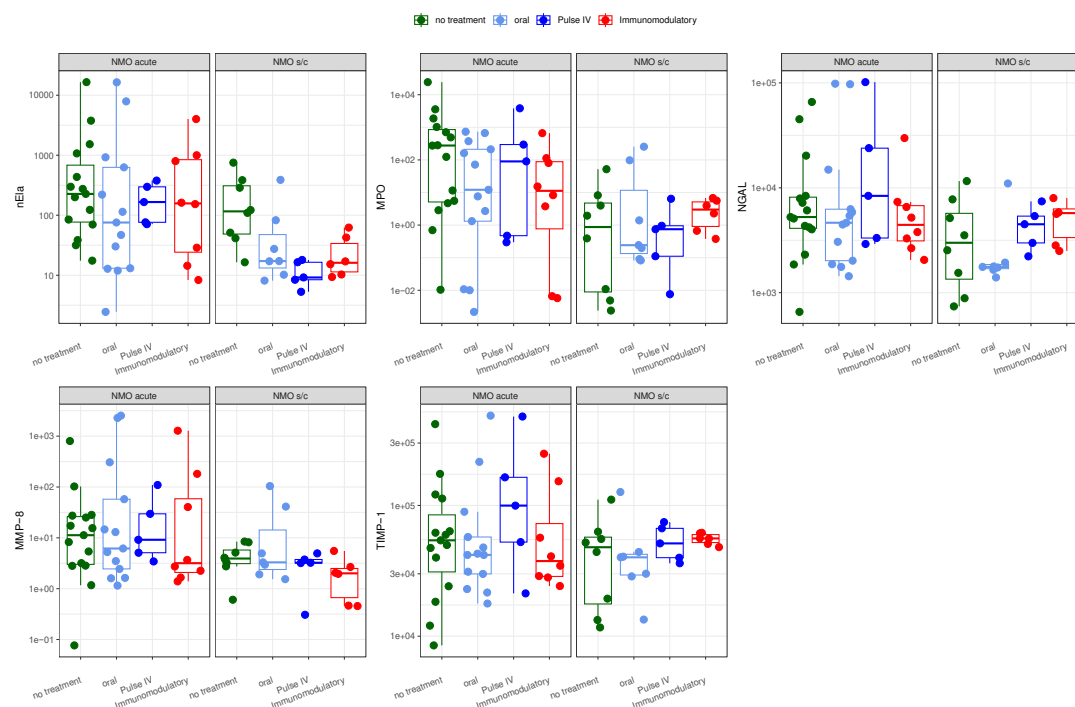
Abbreviations: CXCL13: C-X-C motif chemokine 13; GFAP: glial fibrillary acidic protein; ICAM-1: intercellular adhesion molecule-1; INDC: inflammatory neurological disease control; MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NfL: neurofilament light chain; NGAL: neutrophil gelatinase-associated lipocalin; NMOsD: neuromyelitis optica spectrum disorder; RRMS: relapsing-remitting multiple sclerosis; TIMP-1: tissue inhibitor of metalloproteinase-1; S100B: S100 calcium-binding protein B; s/c: subacute/chronic; SC: symptomatic control; VCAM-1: vascular cell adhesion molecule-1.



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Granulocyte markers identify acute NMOsD

### Supplemental Figure 1B: Effect of immunomodulatory therapy prior to lumbar puncture on marker levels in NMOsD patients



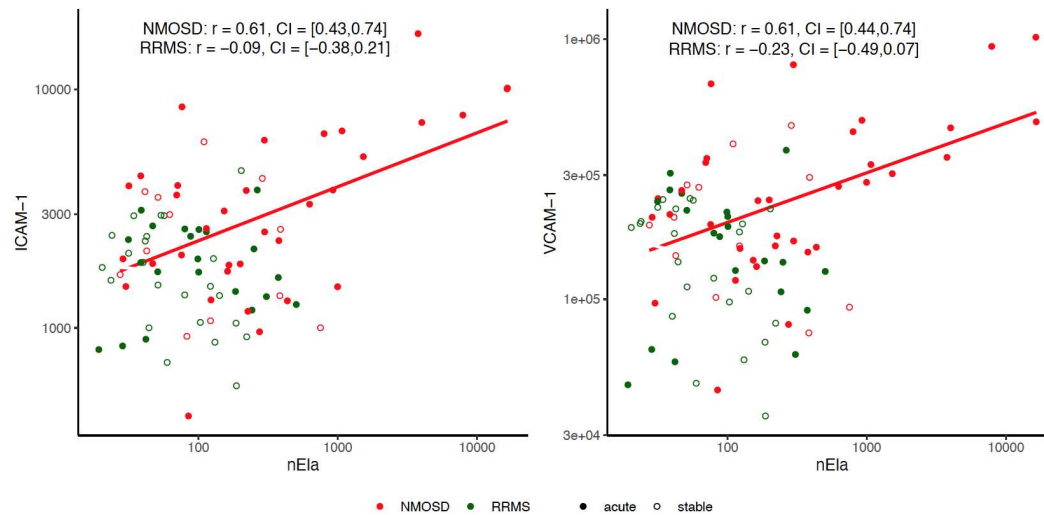
Boxplot of biomarker levels on log-scale (pg/ml) in acute and s/c NMOsD patients, subcategorised for 'no treatment' (●), 'oral steroids only' (●), 'pulse iv steroids' (●), and 'steroids + immunomodulatory therapy' (●).

Abbreviations: MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NGAL: neutrophil gelatinase-associated lipocalin; NMOsD: neuromyelitis optica spectrum disorder; TIMP-1: tissue inhibitor of metalloproteinase-1.

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Granulocyte markers identify acute NMOSD

### Supplemental Figure 2. Correlation of levels of elastase with ICAM-1 and VCAM-1 in pooled cohorts of NMOSD and RRMS patients.



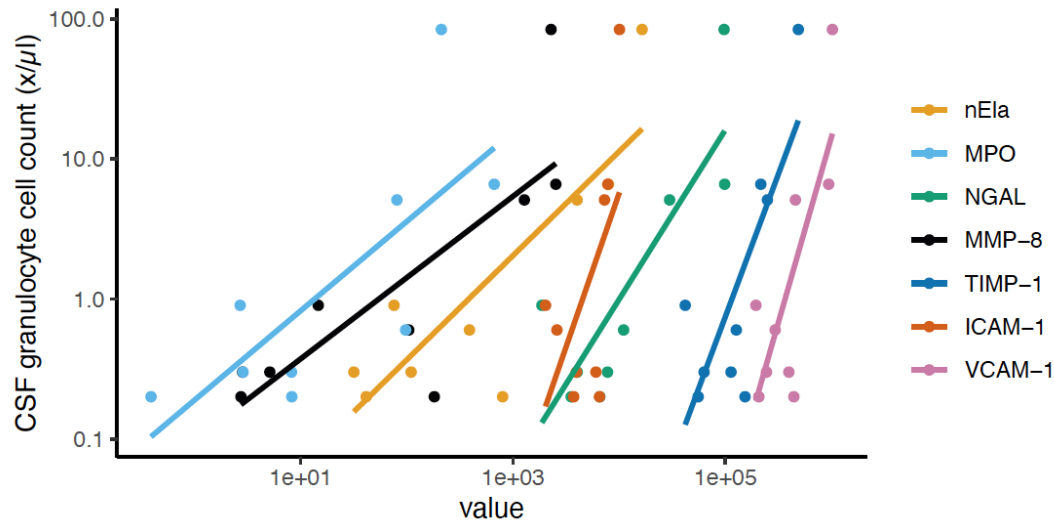
CSF samples from patients (acute (●) and s/c (○) phase, without and with corticosteroid pretreatment) with quantifiable levels of nEla were correlated with levels of ICAM-1 and VCAM-1, respectively. Biomarker values are medians [IQR] in pg/ml. NMOSD: (red), RRMS: (green). Regression line is derived from samples of NMOSD patients with and without corticosteroid pretreatment. The exclusion of NMOSD patients with iv corticosteroid pre-treatment produced virtually identical regression line characteristics: ICAM-1:  $r=0.65$ ,  $CI=[0.47, 0.77]$ ; VCAM-1:  $r=0.72$ ,  $CI=[0.57, 0.82]$ . Regression line characteristics from NMOSD patients with iv or oral corticosteroid pre-treatment excluded were: ICAM-1:  $r=0.56$ ,  $CI=[0.30, 0.75]$ ; VCAM-1:  $r=0.46$ ,  $CI=[0.16, 0.68]$ .

Abbreviations: CI: confidence interval; ICAM-1: intercellular adhesion molecule-1; nEla: neutrophil elastase; NMOSD: neuromyelitis optica spectrum disorder; RRMS: relapsing-remitting multiple sclerosis; VCAM-1: vascular cell adhesion molecule-1.

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**Supplemental Figure 3. Correlation of CSF granulocyte cell count  $\geq 1$  with biomarkers in discovery set NMOSD patients with and without corticosteroid pre-treatment**



Biomarker values are in pg/ml on the x-axis. There was a strong (Rho 0.60-0.79) correlation of cell-specific GAM levels in those nine NMOSD patients with a CSF granulocyte cell count  $\geq 1$  (Rho, p-value: nEla =0.70, 0.037; MPO: 0.70, 0.037; MMP-8: 0.76, 0.018; NGAL: 0.69, 0.040), for TIMP-1 (0.60, 0.090) set significance levels were not reached.

In addition, strong correlation of CSF granulocyte cell count was present for S100B (0.77, 0.014) and GFAP (0.68, 0.044), and a moderate, but not significant correlation (Rho 0.40-0.59) for VCAM-1 (0.59, 0.096) and for ICAM-1 (0.50, 0.166). NfL and CXCL13 showed no correlation with the CSF granulocyte cell count.

Abbreviations: CXCL13: C-X-C motif chemokine 13; GFAP: glial fibrillary acidic protein; ICAM-1: intercellular adhesion molecule-1; MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NfL: neurofilament light chain; NGAL: neutrophil gelatinase-associated lipocalin; NMOSD: neuromyelitis optica spectrum disorder; TIMP-1: tissue inhibitor of metalloproteinase-1; S100B: S100 calcium-binding protein B; VCAM-1: vascular cell adhesion molecule-1.

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Granulocyte markers identify acute NMOSD

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Number of samples	20	15		25	
nEla	114.8 [44.9, 670.8]	94.8 [49.8, 255.7]	1.000	13.2 [9.2, 17.6]	<0.001
MPO	7.9 [2.8, 73.7]	6.1 [2.5, 55.7]	0.610	1.0 [0.2, 2.2]	<0.001
NGAL	6410.5 [5173.0, 17147.5]	9479.0 [5188.0, 15685.0]	0.499	3751.0 [2221.0, 5111.0]	<0.001
MMP-8	8.7 [3.3, 127.3]	2.7 [2.3, 6.4]	0.043	2.2 [1.6, 2.7]	<0.001
MMP-9	270.2 [37.3, 2195.0]	9850.5 [832.3, 51865.4]	0.003	30.8 [20.3, 40.1]	0.006
TIMP-1	62660.0 [51160.0, 155750.0]	82970.0 [54600.0, 131450.0]	0.633	37490.0 [35530.0, 44650.0]	<0.001
GFAP	33958.8 [10730.5, 611223.2]	12125.3 [9313.4, 25050.4]	0.131	9818.0 [6725.0, 11551.7]	0.002
S100B	249.2 [186.0, 496.6]	301.1 [222.8, 402.4]	0.755	248.0 [175.4, 287.0]	0.230
NfL	1732.0 [1134.8, 3696.9]	1278.3 [448.4, 5334.6]	0.188	436.8 [316.3, 517.2]	<0.001
ICAM-1	3697.5 [2004.5, 6224.5]	4862.0 [3323.0, 9052.0]	0.131	2066.0 [1744.0, 2441.0]	0.004
VCAM-1	266900.0 [226175.0, 464200.0]	353000.0 [275600.0, 449000.0]	0.400	157400.0 [133700.0, 209000.0]	<0.001
CXCL13	11.4 [3.9, 47.9]	8.5 [2.5, 84.9]	0.877	0.6 [0.5, 0.9]	<0.001

Biomarker values are medians [IQR] in pg/ml. P values  $\leq 0.05$  are with green background.<sup>‡</sup>P of comparison of acute NMOSD with SC.

Abbreviations: CXCL13: C-X-C motif chemokine 13; GFAP: glial fibrillary acidic protein; ICAM-1: intercellular adhesion molecule-1; INDC: inflammatory neurological disease control; 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; S100B: S100 calcium-binding protein B; SC: symptomatic control; TIMP-1: tissue inhibitor of metalloproteinase-1; VCAM-1: vascular cell adhesion molecule-1.

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**Supplemental Table 2. GAM levels in all NMOSD patients with and without corticosteroid pre-treatment**

Corticosteroid pre-treatment (N)	No 23	Yes 44	P
Days between disease exacerbation and lumbar puncture; median [IQR]	13.0 [6.0, 43.5]	13.0 [7.0, 44.2]	0.982
nEla	199.7 [60.6, 409.9]	36.4 [13.0, 179.4]	0.006
MPO	8.2 [1.3, 385.3]	3.8 [0.3, 91.6]	0.214
NGAL	5095.0 [2423.0, 7837.0]	4480.0 [2180.0, 6760.9]	0.870
MMP-8	5.4 [3.0, 16.4]	3.6 [2.0, 18.4]	0.499
TIMP-1	52020.0 [21725.0, 63212.0]	45870.0 [30832.5, 63017.5]	0.891
S100B	225.3 [159.9, 606.2]	225.9 [182.7, 309.0]	0.900
ICAM-1	3531.0 [1305.5, 4488.0]	2312.5 [1826.0, 3772.5]	0.932
VCAM-1	230900.0 [159450.0, 31685.0]	212000.0 [158250.0, 305425.0]	0.716

Biomarker values are medians [IQR] in pg/ml. P values  $\leq 0.05$  are with green background.

Abbreviations: ICAM-1: intercellular adhesion molecule-1; MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NGAL: neutrophil gelatinase-associated lipocalin; NMOSD: neuromyelitis optica spectrum disorder; S100B: S100 calcium-binding protein B; TIMP-1: tissue inhibitor of metalloproteinase-1; VCAM-1: vascular cell adhesion molecule-1.

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**Supplemental Table 3. Association between EDSS and biomarkers in samples of NMOSD patients with and without corticosteroid pre-treatment**

Marker	all NMOSD N=67		acute NMOSD N=41		s/c NMOSD N=26	
	Rho [CI]	p	Rho [CI]	p	Rho [CI]	p
nEla	0.38 [0.16-0.57]	0.001	0.24 [-0.11-0.48]	0.135	0.41 [0.03-0.69]	0.036
MPO	0.31 [0.08-0.52]	0.010	0.20 [-0.1-0.48]	0.213	0.21 [-0.19-0.56]	0.293
NGAL	0.46 [0.24-0.63]	<0.001	0.50 [0.22-0.70]	<0.001	0.27 [-0.13-0.60]	0.181
MMP-8	0.40 [0.18-0.59]	<0.001	0.40 [0.11-0.63]	0.009	0.27 [-0.13-0.60]	0.178
TIMP-1	0.35 [0.12-0.54]	0.004	0.39 [0.10-0.63]	0.011	0.28 [-0.12-0.60]	0.168
ICAM-1	0.39 [0.16-0.57]	0.002	0.47 [0.19-0.68]	0.002	0.18 [-0.22-0.53]	0.375
VCAM-1	0.38 [0.15-0.57]	0.001	0.46 [0.17-0.67]	0.003	0.14 [-0.26-0.50]	0.501

P values  $\leq 0.05$  are with green background.

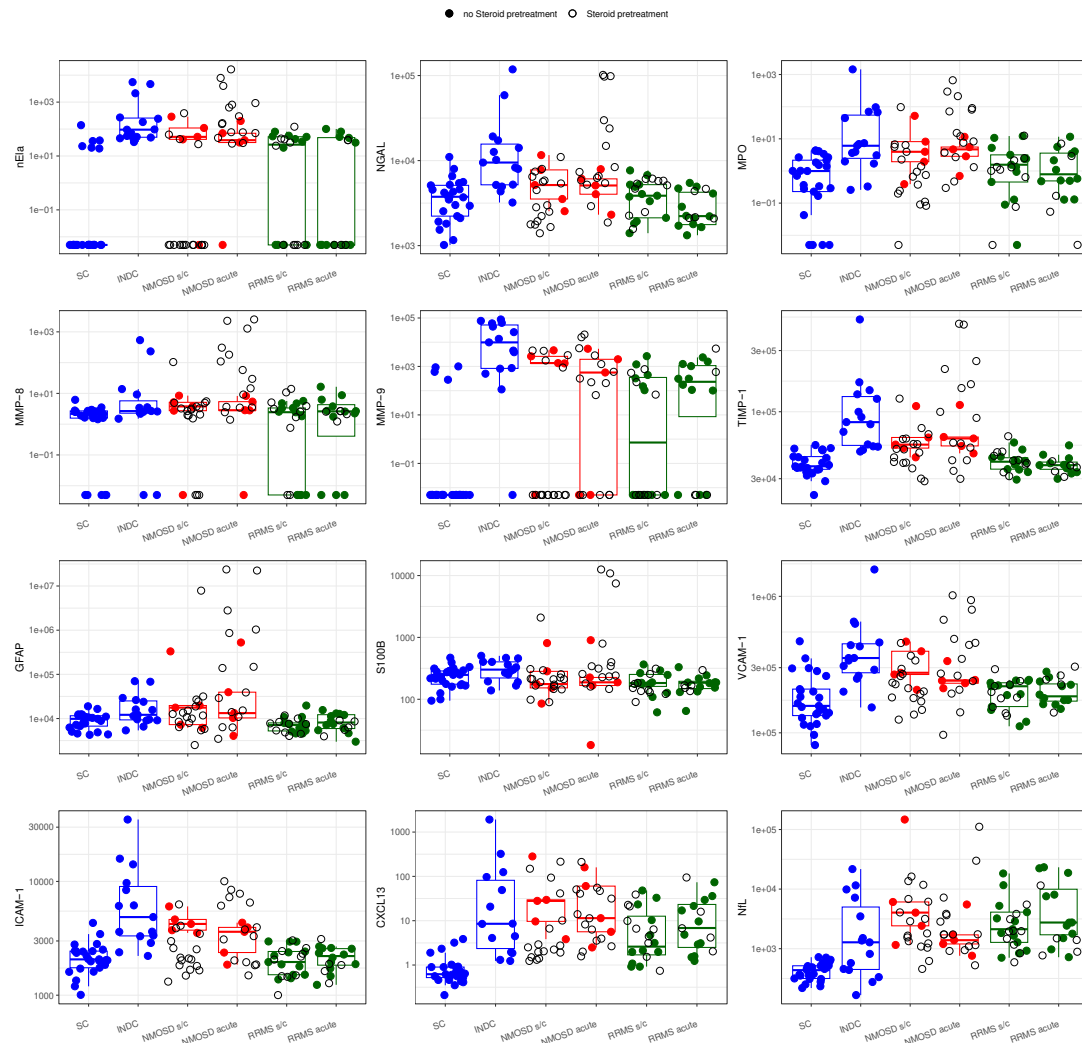
Abbreviations: CXCL13: C-X-C motif chemokine 13; GFAP: glial fibrillary acidic protein; ICAM-1: intercellular adhesion molecule-1; MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NGAL: neutrophil gelatinase-associated lipocalin; NfL: neurofilament light chain; NMOSD: neuromyelitis optica spectrum disorder; RRMS: relapsing-remitting multiple sclerosis; TIMP-1: tissue inhibitor of metalloproteinase-1; S100B: S100 calcium-binding protein B; VCAM-1: vascular cell adhesion molecule-1.

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### Supplemental Figure 1A. Biomarker levels in NMOsD, RRMS, SC and INDCs in discovery cohort



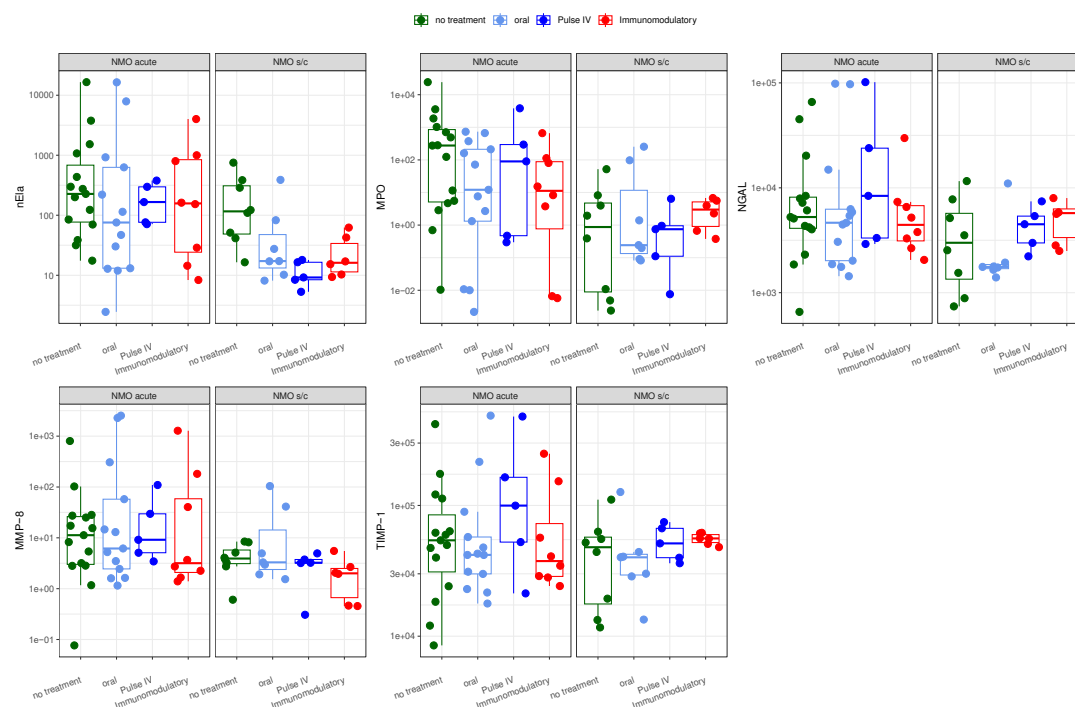
Boxplot of biomarker levels on log-scale (pg/ml) by patient population. Patients with corticosteroid pre-treatment (○) are not used for the boxplot calculations. Note that many patients under corticosteroid treatment in acute NMOsD have values above the 3<sup>rd</sup> quartile/75<sup>th</sup> percentile.

Abbreviations: CXCL13: C-X-C motif chemokine 13; GFAP: glial fibrillary acidic protein; ICAM-1: intercellular adhesion molecule-1; INDC: inflammatory neurological disease control; MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NfL: neurofilament light chain; NGAL: neutrophil gelatinase-associated lipocalin; NMOsD: neuromyelitis optica spectrum disorder; RRMS: relapsing-remitting multiple sclerosis; TIMP-1: tissue inhibitor of metalloproteinase-1; S100B: S100 calcium-binding protein B; s/c: subacute/chronic; SC: symptomatic control; VCAM-1: vascular cell adhesion molecule-1.

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### Supplemental Figure 1B: Effect of immunomodulatory therapy prior to lumbar puncture on marker levels in NMOsD patients



Boxplot of biomarker levels on log-scale (pg/ml) in acute and s/c NMOsD patients, subcategorised for 'no treatment' (●), 'oral steroids only' (●), 'pulse iv steroids' (●), and 'steroids + immunomodulatory therapy' (●).

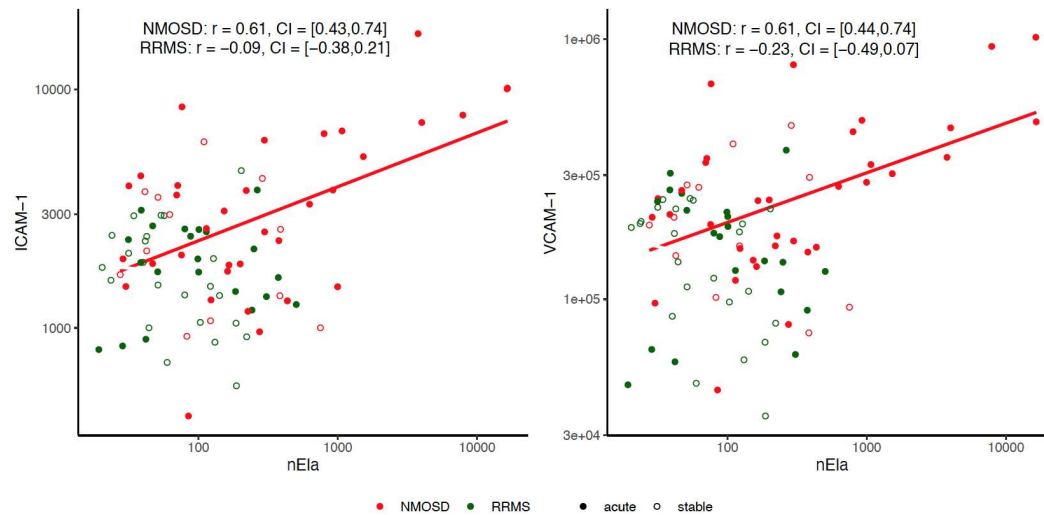
Abbreviations: MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NGAL: neutrophil gelatinase-associated lipocalin; NMOsD: neuromyelitis optica spectrum disorder; TIMP-1: tissue inhibitor of metalloproteinase-1.



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### Supplemental Figure 2. Correlation of levels of elastase with ICAM-1 and VCAM-1 in pooled cohorts of NMOSD and RRMS patients.



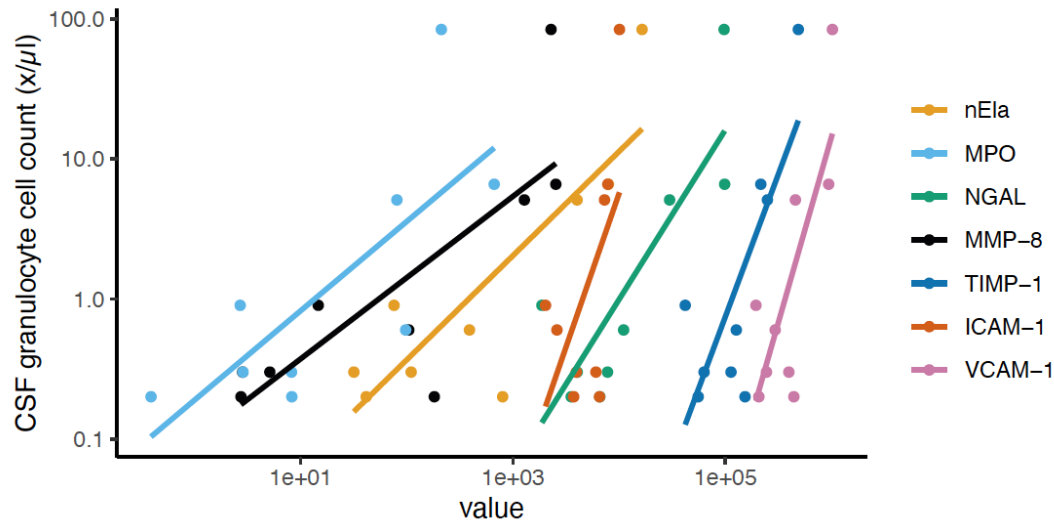
CSF samples from patients (acute (●) and s/c (○) phase, without and with corticosteroid pretreatment) with quantifiable levels of nEla were correlated with levels of ICAM-1 and VCAM-1, respectively. Biomarker values are medians [IQR] in pg/ml. NMOSD: (red), RRMS: (green). Regression line is derived from samples of NMOSD patients with and without corticosteroid pretreatment. The exclusion of NMOSD patients with iv corticosteroid pre-treatment produced virtually identical regression line characteristics: ICAM-1:  $r=0.65$ ,  $CI=[0.47, 0.77]$ ; VCAM-1:  $r=0.72$ ,  $CI=[0.57, 0.82]$ . Regression line characteristics from NMOSD patients with iv or oral corticosteroid pre-treatment excluded were: ICAM-1:  $r=0.56$ ,  $CI=[0.30, 0.75]$ ; VCAM-1:  $r=0.46$ ,  $CI=[0.16, 0.68]$ .

Abbreviations: CI: confidence interval; ICAM-1: intercellular adhesion molecule-1; nEla: neutrophil elastase; NMOSD: neuromyelitis optica spectrum disorder; RRMS: relapsing-remitting multiple sclerosis; VCAM-1: vascular cell adhesion molecule-1.

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**Supplemental Figure 3. Correlation of CSF granulocyte cell count  $\geq 1$  with biomarkers in discovery set NMOSD patients with and without corticosteroid pre-treatment**



Biomarker values are in pg/ml on the x-axis. There was a strong (Rho 0.60-0.79) correlation of cell-specific GAM levels in those nine NMOSD patients with a CSF granulocyte cell count  $\geq 1$  (Rho, p-value: nEla = 0.70, 0.037; MPO: 0.70, 0.037; MMP-8: 0.76, 0.018; NGAL: 0.69, 0.040), for TIMP-1 (0.60, 0.090) set significance levels were not reached.

In addition, strong correlation of CSF granulocyte cell count was present for S100B (0.77, 0.014) and GFAP (0.68, 0.044), and a moderate, but not significant correlation (Rho 0.40-0.59) for VCAM-1 (0.59, 0.096) and for ICAM-1 (0.50, 0.166). NfL and CXCL13 showed no correlation with the CSF granulocyte cell count.

Abbreviations: CXCL13: C-X-C motif chemokine 13; GFAP: glial fibrillary acidic protein; ICAM-1: intercellular adhesion molecule-1; MMP: matrix metalloproteinase; MPO: myeloperoxidase; nEla: neutrophil elastase; NfL: neurofilament light chain; NGAL: neutrophil gelatinase-associated lipocalin; NMOSD: neuromyelitis optica spectrum disorder; TIMP-1: tissue inhibitor of metalloproteinase-1; S100B: S100 calcium-binding protein B; VCAM-1: vascular cell adhesion molecule-1.