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ORIGINAL COMMUNICATION

Antibodies against neural antigens in patients with acute stroke: joint results of three independent cohort studies

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Abstract

Background and purpose Ischemic stroke (IS) and hemorrhagic stroke (HemS) typically lead to a breakdown of the blood–brain barrier with neural antigen presentation. This presentation could potentially generate destructive auto-immune responses. Pre-existing antineuronal and antiglial antibodies (AA), predominantly NMDA receptor antibodies, have been reported in patients with stroke. This article summarizes three independent prospective studies, the Lübeck cohort (LC), Barcelona cohort (BC), and Heidelberg cohort (HC), exploring the frequency and clinical relevance of AA in patients with acute stroke (AS).

Methods In all cohorts together, 344 consecutive patients admitted with AS (322 × IS, 22 × HemS) were screened for AA in serum at admission. Clinical outcome parameters as well as a second AA screening were available at 30 days in the LC or at 90 days in the BC. A control group was included in the BC (20 subjects free from neurological disease) and the HC (78 neurological and ophthalmological patients without evidence for stroke).

Results The rate of positivity for AA was similar in control subjects and AS patients (13%, 95% CI [7%, 22%] vs. 13%, 95% CI [10%, 17%]; $p = 0.46$) with no significant difference between cohorts (LC 25/171, BC 12/75, HC 9/98). No patient had developed new AA after 30 days, whereas 2 out of 60 patients had developed new AA after 90 days. AA positive patients did not exhibit significant differences to AA negative patients in stroke subtype (LC, BC), initial stroke severity (BC, LC, HC), infarct volume (BC), and functional status at admission (BC, LC, HC) and follow-up (BC, LC).

Conclusions AS does not induce AA to a relevant degree. Pre-existing AA can be found in the serum of stroke patients, but they do not have a significant association with clinical features and outcomes.

Keywords Stroke · Antineural antibodies · Anti-NMDAR antibodies · Anti-CASPR2 antibodies · Anti-GAD65 antibodies · Anti-aquaporin antibodies

Abbreviations

AA	Antineuronal and antiglial antibodies in serum
APOE4	Apolipoprotein E4
AS	Acute stroke
BBB	Blood–brain barrier
BC	Barcelona cohort
CRS	Coma Rating Scale
DRS	Delirium Rating Scale
DW-MRI	Diffusion-weighted magnetic resonance imaging
FIM	Functional independence measure
GCS	Glasgow Coma Scale
HemS	Hemorrhagic stroke
HC	Heidelberg cohort
IS	Ischemic stroke
LC	Lübeck cohort
MMSE	Mini Mental State Examination
mRS	Modified Rankin Scale
NIHSS	National Institute of Health Stroke Scale

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Introduction

The severity of the initial neurological deficit, which depends on the localization and size of the brain lesion, is a significant determinant of the outcome of acute stroke (AS) patients. However, multiple other factors contribute to the outcome, including well-known clinically apparent complications such as pneumonia and seizures, and other factors related to brain tissue regeneration that are better known at the experimental level. Concerning the latter, there is evidence on immune responses induced by ischemic stroke (IS) [1]. A "stroke-induced immunodeficiency syndrome" has been described that leads to an increased susceptibility to infections [2]. Infections, mainly pneumonia, can increase the morbidity and mortality of patients who have initially survived an AS [3]. In contrast to the damaging effect of infections, it has been debated whether the immunosuppression might at the same time be protective by preventing strong immune reactions against brain tissue [4]. Such reactions can occur due to disruption of the blood–brain barrier (BBB) and consecutive exposition of immune cells to myelin and neuronal antigens [5]. In recent years, multiple antineuronal and antiglial antibodies (AA) with specific clinical syndromes have been identified, reflecting a destructive autoimmune response [6]. These antibodies have been described in patients with diverse diseases, and seem to be pathogenic in certain conditions such as tumors and herpes-simplex virus encephalitis [6, 7]. In stroke patients, Kalev-Zylinska et al. found antibodies against NMDA receptor subunit 2 in 21 of 48 (44%) ischemic stroke patients. However, the detection was based on ELISA and western blot rather than state-of-the art immunohistochemistry with transfected cells, and, therefore, a rather unspecific reaction cannot be excluded [8]. Using immunohistochemistry, Zerche et al. found antibodies against NMDAR1 in 22% of 464 patients with acute ischemic stroke and described a beneficial effect of NMDAR1 autoantibodies on the evolution of the ischemic lesion size in patients that were negative for apolipoprotein E4 (APOE4), but they found the opposite effect of autoantibodies in APOE positive patients [9]. This different effect of autoantibodies depending in APOE4 was attributed to APOE4-dependent differences in blood–brain barrier integrity [9]. Importantly, the immunoglobulin isotype is critical to determine pathogenicity, at least for certain autoantibodies, as shown in antibody-mediated myocarditis [10].

This study aimed to determine the frequency and subtype of autoantibodies against NMDA and a variety of other neuronal and glial antigens in independent cohorts of stroke patients: Lübeck, Germany (LC), Barcelona, Spain (BC) and Heidelberg, Germany (HC) cohorts. We assessed the clinical relevance of AA and re-evaluated the patients to identify any prognostic value of AA. We also searched for a de-novo synthesis of AA after stroke that could suggest a vaccination-like effect induced by AS.

Methods

Clinical data and blood were collected upon formal written consent in accordance with the Declaration of Helsinki and after positive approval of the local ethical committee. The inclusion criteria and general study design of each of the three cohort are summarized in Table 1. All cohorts investigated AA in AS patients (LC $n=171$, BC $n=75$, HC $n=98$) along with different clinical outcome parameters and risk factor assessments. A second AA screening was performed at day 26–30 after AS in LC and at day 90 in BC. As lumbar puncture was not an obligatory step in standard diagnostic workup of stroke, CSF was not available in our patients. Control groups were examined in BC (20 subjects free from neurological disease) and HC (78 neurological and ophthalmological patients without evidence for stroke). BC included patients from the prospective "Immunological Biomarkers in Patients with Acute Ischemic Stroke" study (Clinicaltrials.gov identifier NCT01894529) in which all serum samples (days 0, 1, and 90) were available. Infarct volume was measured in BC patients by diffusion-weighted MRI (DW-MRI) within 72 h of stroke onset in 70/75 patients. In addition, small vessel disease was graded using ARWMC and Fazekas scores, and the number of perivascular spaces and microhemorrhages were quantified. In BC patients, serum samples were collected at three different timepoints (days 0, 1, and 90) from stroke patients and once in control subjects. Plasma concentrations of MMP-2, MMP-3, MMP-7, MMP-9, and MMP-13 were measured at days 0 and 1 using a commercially available ELISA-bases assay (Q-PlexTM human MMP (5-Plex) #340949HU, Quansys Biosciences) as markers of blood–brain barrier breakdown.

Testing for AA

Testing for AA was performed in the same laboratory for the BC, LC, and HC. Detection of antigen–antibody binding pattern and presence of autoantibodies were screened for using commercially available EUROIMMUN BIOCHIP Mosaics™ (EUROIMMUN, Lübeck, Germany) [11]. Briefly, this mosaic consists of frozen sections of rat cerebellum and hippocampus, primate cerebellum, intestine, nerve, and pancreas—each cryosectioned at a thickness of 5 µm. The mosaic also consists of acetone fixed NMDAR and glutamate decarboxylase-65 (GAD65) transfected cells and formalin fixed gamma-aminobutyric acid-B receptor (GABABR), aquaporin-4 (AQP4), leucine-rich, glioma

Table 1 Inclusion criteria and datasets obtained in the three cohorts

Cohort	Luebeck cohort (LC)	Barcelona cohort (BC)	Heidelberg cohort (HC)
Patients included (*n*)	171	75	98
Inclusion criteria	Acute stroke (ischemic or hemorrhagic) with functionally relevant impairment (NIHSS > 2)	Ischemic stroke admitted within 6 h of symptom onset, NIHSS > 2 and treated with systemic or intraarterial thrombolysis	Acute ischemic stroke
Exclusion criteria	Pre-existing dementia	None	None
Clinical assessment	NIHSS, mRS, Barthel Score, GCS, MMSE, DRS, FIM, CRS	NIHSS, mRS	NIHSS, mRS
Risk factor assessment	Diabetes mellitus, arterial hypertension, atrial fibrillation, smoking, hypercholesterolemia, previous stroke, coronary heart disease, peripheral arterial disease	/	Diabetes mellitus, arterial hypertension, atrial fibrillation, smoking, hypercholesterolemia, previous stroke, coronary heart disease, peripheral arterial disease, ratio waist/hip circumference
Stroke type assessment	Ischemic vs. hemorrhagic, right-sided lesion	Infarct volume with DW-MRI within 72 h of stroke onset (70/75 patients), small vessel disease grading with ARWMC and Fazekas scores, quantification of perivascular spaces and microhemorrhages	/
Lab exam	AA, WBC, Hb, Na, CK, creatinine, CRP	AA, plasma concentrations of MMP-2, MMP-3, MMP-7, MMP-9, MMP-13	AA, serum glucose, HbA1c
Patients lost to follow-up (*n*)	35 (16 due to death, 19 due to traveling, decline to follow-up or logistical reasons)	0	0
Time of follow-up assessment	Day 26–30 with 2nd AA screening	Day 1, day 90, with 2nd AA screening	Day 90 without 2nd AA screening
Control group	/	20 Age-matched control subjects free from acute neurological disease	78 Neurological and ophthalmological patients without evidence for stroke

NIHSS National Institute of Health Stroke Scale, *mRS* Modified Rankin Scale, *GCS* Glasgow Coma Scale, *MMSE* Mini Mental State Examination, *DRS* Delirium Rating Scale, *FIM* functional independence measure, *CRS* Coma Rating Scale, *ARWMC* age related white matter changes, *AA* antineural and antiglial antibodies, *WBC* white blood cell count, *Hb* hemoglobin, *CK* creatine kinase, *CRP* C-reactive protein, *MMP* matrix metalloproteinase

inactivated 1 (LGI1) and contactin-associated protein-like 2 (CASPR2) transfected cells as substrates using HEK293 cell line for transfection. Indirect immunofluorescence (IFT) was done with a serum dilution of 1:10 and using a secondary anti-human IgG isotype antibody conjugated with FITC. Cells or tissue sections were used as antigen substrates and the results were read in a EuroStar II/Zeiss Axioskop 2 microscope. Reading of immunofluorescence pattern and microscopy was performed within 24 h of incubation by two independent researchers.

Statistical analysis

Continuous variables were reported as mean ± standard deviation or median with interquartile ranges. Proportions of positive AA findings were expanded by 95% confidence intervals generated by Clopper–Pearson intervals. According to the type of data, we used the appropriate tests to assess significant differences between groups. Categorical variables were compared with Chi-square test or Fisher exact tests. For ordinally scaled data, Wilcoxon rank-sum test was used; for continuous data the Student's *t* test, one-way analysis of variance, Mann–Whitney, or Kruskal–Wallis was applied. Correlations were assessed with Spearman coefficient. Ordinal regression models were used to assess the effects of autoreactivity on functional outcome at 90 days adjusted for variables associated with the presence of autoreactivity and outcome with $p < 0.05$ on univariate analysis. The analysis was performed using SPSS (v22; IBM, Armonk, NY) or MATLAB® R2018a (The MathWorks, Inc., USA), and the level of significance was established at the 0.05 level (2-sided).

Results

Autoreactivity was found in stroke patients and controls with a range of different antineuronal and other antibodies (Fig. 1).

Proportion of AA seropositive stroke patients

Figure 2a illustrates the frequency of AA seropositive patients pooled across all three studies. After testing 344 AS patients, pre-existing AA were found in 46 patients (13%, 95% CI [10%, 17%], LC 25/171, BC 12/75, HC 9/98), which was not different ($p = 0.46$) from the proportion of AA positive controls (13 out of 98, 95% CI [10%, 17%]). Figure 2b displays the proportions AA positive patients in each cohort with no significant differences across cohorts.

Figure 3, and Table S1 provide data on the 61 subjects with positive AA screening. The most frequent prevalent

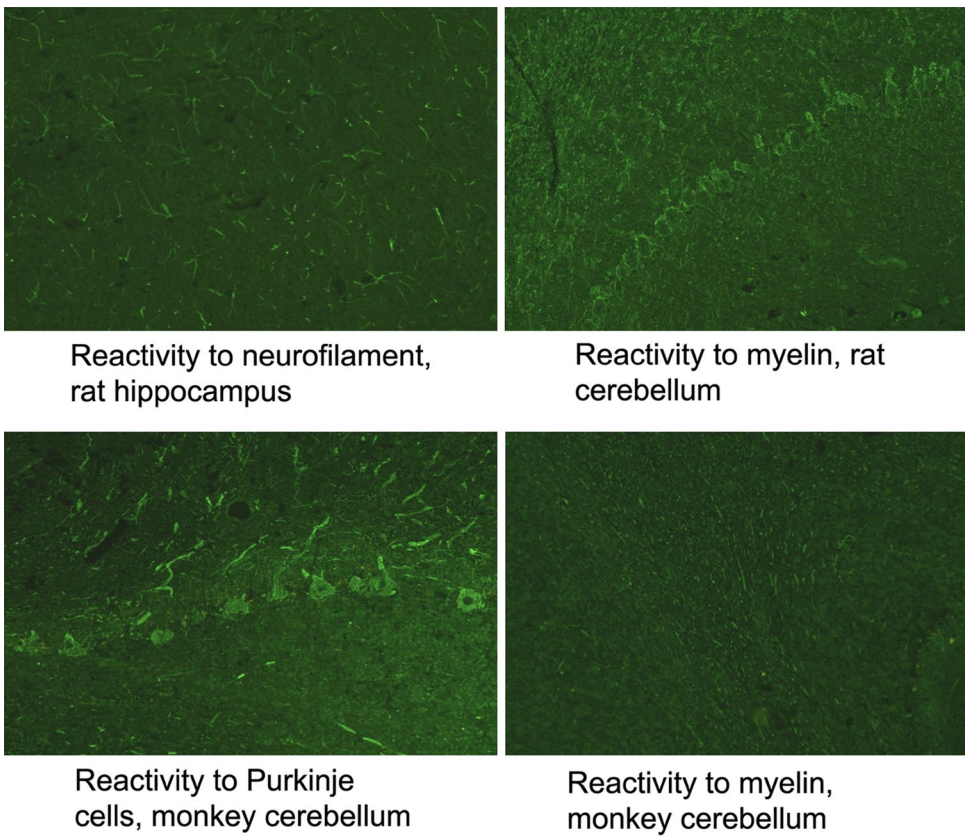

Fig. 1 Examples of autoreactivity in serum from stroke patients (BC)

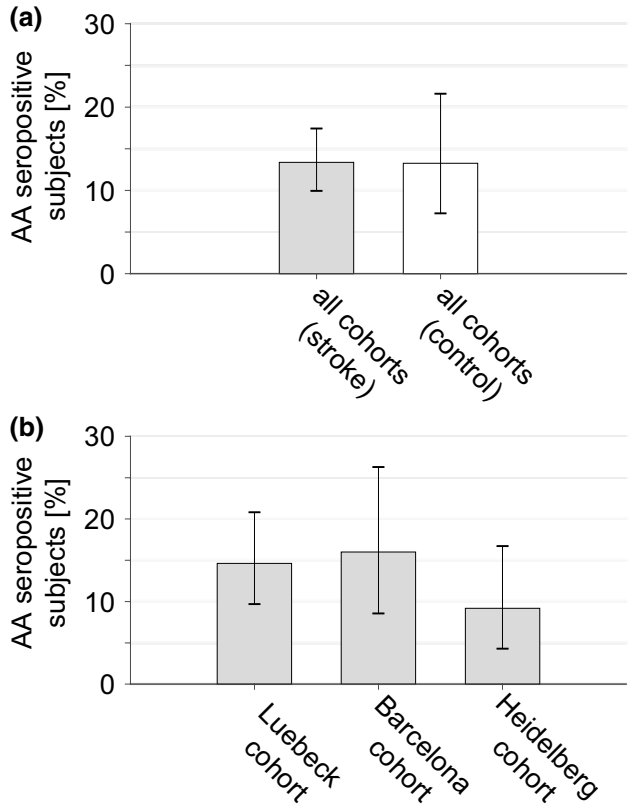

Fig. 2 Proportion of AA positive subjects pooled across cohorts (**a**, $n=344$ patients with AS, $n=98$ control patients) and within each cohort (**b**). Error bars denote 95% CI based on Clopper–Pearson intervals

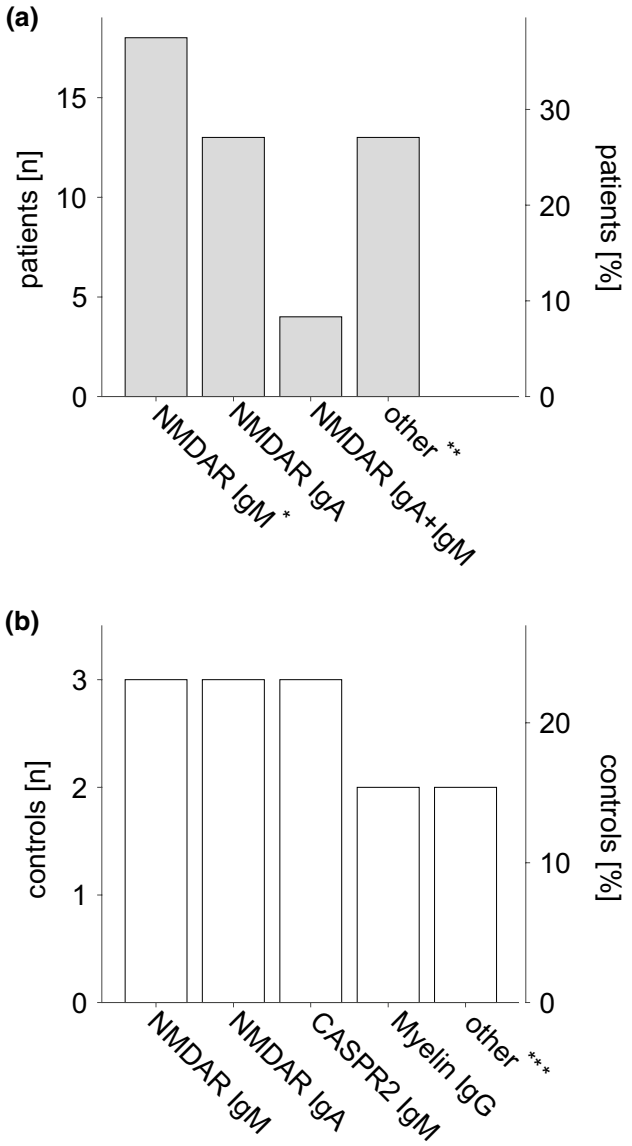

Fig. 3 Distribution of AA types in patients with AS (**a**) and control patients (**b**). (*2 of 18 patients were positive only in follow-up after 30 days indicating de-novo synthesis. **Singular occurrences of CASPR2 IgA, CASPR2 IgG, GAD65 IgG, Aquaporin-4 IgA, NMDAR IgM + NF IgM, NF IgM, PCA2 IgG, molecular layer IgG, CASPR2 IgG, CASPR2 IgM, glycine receptor IgA, myelin IgG, myelin IgA and NMDAR IgG. ***Singular occurrences of NMDAR IgG + IgM and NMDAR IgG)

antibodies in both AS and control patients were NMDAR IgM and/or NMDAR IgA ($n=32$ in AS patients, $n=6$ in controls), followed by CASPR2 IgG and/or CASPR2 IgM or CASPR2 IgA ($n=3$ in AS patients, $n=3$ in controls) and Myelin IgG or IgA ($n=2$ in AS patients, $n=1$ in controls). NMDAR IgG were found in one AS and two controls. Two AS patients had antibodies against neurofilament. In addition, we found singular occurrences of antibodies against Aquaporin-4 IgA, GAD65 IgG, GlycinR IgA, Molecular layer IgG and PCA2 IgG. No AS patient developed de-novo antibodies after 4 weeks (LC), whereas 2 AS patients developed de-novo antibodies against NMDAR IgM at follow-up at day 90 (BC) (Fig. 3).

The clinical variables obtained in the different studies are summarized in Table S2. When available from more than one cohort, data from all patients were pooled. No significant differences were observed concerning risk factors, stroke type, routine laboratory results, and clinical outcome when comparing AA positive and AA negative patients.

Radiological characteristics of AA positive and AA negative patients were not significantly different (BC, Table S3). Higher levels of some MMPs were associated with a more significant burden of small vessel disease on MRI. Levels of MMP-2 and MMP-3 at baseline were positively correlated to higher scores in the ARWMC scale and more perivascular spaces and greater MMP-9 levels at day 1 were also correlated with the number of microhemorrhages However, there were no significant differences between patients and controls in any of the MMP levels. In addition, the levels of all MMPs were also similar in patients with positive AA compared to patients with no AA (data not shown).

Discussion

This multicenter study screened 344 AS patients for AA and found a frequency of 13% AA seropositive patients. Predominantly, NMDAR IgM and IgA were found, but also a variety of other known AA subtypes. In 98 control patients, this frequency was 13% with a similar distribution of AA subtypes.

A recent study compared three different myelin oligodendrocyte glycoprotein-IgG cell-based assays and showed a higher sensitivity and specificity of live cell-based assays as opposed to fixed cell-based assays similar to our approach [12]. In theory, therefore, there might have been false-negative and false-positive results in our study. However, this would predominantly affect the data on follow-up which only found de-novo AA in 2 out of 60. We, therefore, doubt that a testing with an additional assay would have changed the results of our study relevantly.

When limiting the patients to NMDAR positive patients, the percentage of positive anti-NMDAR antibodies in our stroke patients of (11%) was lower than in the study by Zerche et al. that described antibodies in 22% of the patients [9]. Confirming the results of this study, we found no significant differences in clinical severity and risk factors. Zerche et al. found differences in ischemic lesion size evolution from day 1 to day 7 in ischemic strokes of the middle cerebral artery territory, depending on APOE4 carrier status. We cannot exclude that this effect is also existent in our patients, since the design of our study was different. Our studies had broader inclusion criteria, e.g., also included hemorrhagic strokes (LC) and ischemic strokes in territories other than MCA. In this heterogeneous patient group, the existence of anti-neuronal antibodies did not have a relevant influence on clinical outcome in the acute phase and at follow-up. However, we cannot exclude that patients with anti-NMDAR antibodies have different outcomes depending on their APOE4 carrier status, since our study did not assess the latter. Interestingly, it has been reported that the APOE genotype alters the immunoglobulin subtypes in mice, thus showing an influence of APOE genotype on the antibody responses of the immune system [13].

Similar to the study by Zerche et al. the frequency of NMDAR IgM and IgA autoantibodies found in our patients was almost two magnitudes higher than the frequency of NMDAR IgG autoantibodies [9]. Of note, NMDAR IgM and IgA autoantibodies are different from autoantibodies found in anti-NMDA-receptor encephalitis, which are of the IgG-subclass [14]. The isotype of antibodies is essential for its possible pathogenic function. For instance, in antibody-mediated myocarditis in mice, IgG anti-myosin antibodies induce the disease, whereas IgM anti-myosin antibodies do not, but the latter can become pathogenic after undergoing heavy chain class switching to IgG [10]. Likewise, IgG NMDAR antibodies have a larger pathogenic capacity than IgM or IgA antibodies. The former reduce NMDAR levels and are specific for anti-NMDAR encephalitis, whereas IgA or IgM antibodies do not alter receptor levels and have been reported in other diseases [15]. In contrast, another study reported that all naturally occurring autoantibodies against the NMDA receptor subunit NR1 have pathogenic potential irrespective of epitope and immunoglobulin class [16]. In our stroke cohorts, IgG NMDAR antibodies were only detected in one patient with hemorrhagic stroke, whereas most patients showed IgA or IgM NMDAR antibodies. The potential pathogenicity of IgM and IgA antibodies against myelin-associated proteins is also unclear. For instance, MOG antibodies occur in 19% of multiple sclerosis patients with MOG-IgG-associated disease, but they do not seem to play a relevant clinical role [17]. However, IgM antibodies against specific molecules such as phosphorylcholine seem to have prognostic value for adverse cardiovascular events as assessed in 1062 patients with stable coronary heart disease [18]. Increased permeability of the BBB might facilitate the access of circulating antibodies to the injured brain tissue. Therefore, it is interesting to relate the presence of AA with BBB dysfunction. In one of our patient cohorts (BC), we measured plasma MMPs as indicators of BBB disruption [19]. However, we did not find a relation between increased MMP levels, the presence of AA, and clinical outcome. Nevertheless, future studies in larger cohorts of stroke patients are needed to clarify whether the presence of IgM and IgA AA may have any prognostic value in stroke patients.

Our study adds to the existing literature on NMDAR antibodies in non-encephalitic neurological diseases. In a recent study, Hopfner et al. screened 296 patients with Parkinson disease and found 13% with NMDAR IgA/IgM antibodies as opposed to 22% in controls free of neuropsychiatric disease [20]. Similar to our findings, seropositivity was not associated with clinical or epidemiological characteristics of Parkinson patients [20]. Another study found NMDAR IgA/IgM antibodies in 7 of 90 patients with dementia and 0 of 50 patients with Schizophrenia [15]. Dahm et al. found NMDAR antibodies in 8% of patients with Parkinson, in 9% of patients with Schizophrenia, in 5% of patients with amyotrophic laterals sclerosis, and in 9% of healthy controls [21].

IgG antibodies against NMDAR have been described in cases of autoimmune encephalitis secondary to a herpes-simplex encephalitis [22]. Two of the sub-studies searched for a de-novo production of AA in patients with acute stroke, which would prove a vaccination effect caused by tissue damage. After 4 weeks (LC), no de-novo synthesized antibodies were detected, whereas after 90 days (BC), two

patients had new AA against NMDAR of IgM isotype. Studies on CSF in stroke patients have found intrathecal immunoglobulin synthesis in stroke patients [23, 24]. However, in our study, AA seropositive patients were not more likely to have had a previous stroke than AA seronegative patients were. Taken together, our data thus argues against a significant de-novo production of AA caused by acute stroke. Nevertheless, this does not exclude a secondary development of AA. A false-negative result could be due to our limited sample size. The effect of antibody production might only occur in a tiny proportion of patients. Second, antibody production might be delayed. Third, it is possible that antibodies were only present within the cerebrospinal fluid compartment.

Conclusions

While AA can be detected in a significant proportion of stroke patients, they do not seem to be specific of stroke, and they are mainly of the IgM and IgA subtypes. Although we could not detect a significant prognostic value of AA in stroke patients, the results need validation in larger cohorts and investigation of antibodies against neural antigens in CSF.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical standards All human studies must state that they have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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