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Original article

Investigation of neuronal auto-antibodies in children diagnosed with epileptic encephalopathy of unknown cause

Pınar Tekturk [a,*], Betul Baykan [a], Ece Erdag [b], Sian Peach [c], Mine Sezgin [a], Zuhal Yapici [a], Cem İsmail Küçükali [b], Angela Vincent [c], Erdem Tuzun [b]

[a] Istanbul University, Istanbul Faculty of Medicine, Department of Neurology, Units of Child Neurology and Clinical Neurophysiology, Istanbul, Turkey
[b] Istanbul University, Institute of Experimental Medical Research, Department of Neuroscience, Istanbul, Turkey
[c] University of Oxford, Nuffield Department of Clinical Neurosciences, John Radcliffe Hospital, Oxford, United Kingdom

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Abstract

Aim: Cryptogenic forms of epileptic encephalopathies (EE) with their well-known features of drug-resistance, mental deterioration and partial response to immunotherapies are ideal candidates for screening for neuronal autoantibodies (NAA).

Method: Fifty consecutive pediatric patients with a diagnosis of EE of unknown cause were included. Nine NAAs were tested by ELISA, RIA or cell-based assays. Clinical features of seronegative and seropositive patients were compared.

Results: NAAs were found in 7/50 (14%) patients. They were N-methyl-D-aspartate receptor in two (4%), glycine receptor in two (4%), contactin-associated protein-like 2 in one (2%), glutamic acid decarboxylase in one (2%) and type A gamma aminobutyric acid receptor in one patient (2%). Furthermore, serum IgGs of two patients negative for well-characterized NAAs, showed strong reactivity with the uncharacterized membrane antigens of live hippocampal neurons. There were no significant differences between seropositive and seronegative patients by means of epilepsy duration, anti-epileptic drug resistance, EE type, types of seizures, seizure frequencies, EEG features or coexisting autoimmune diseases. Some seropositive patients gave good-moderate response to immunotherapy.

Discussion: Potential clues for the possible role of autoimmunity in seropositive patients with EE were atypical prognosis of the classical EE type, atypical progression and unusual neurological findings like dyskinesia.

Keywords: Epileptic encephalopathy; Neuronal auto-antibodies; West syndrome; Lennox-Gastaut syndrome; Immune-mediated epilepsy

1. Introduction

As an exciting development in recent years, various neuronal auto-antibodies (NAA) were detected in many patients with autoimmune encephalitis often associated with drug-resistant epilepsy and status epilepticus [1]. Seizures associated with NAA are divided into two main clinical groups in adults and children [2,3]. While in adults, these antibodies mostly cause epileptic syndromes that are associated with temporal lobe inflammation, in children, they are often associated with autoimmune encephalopathies that present with diffuse involvement of the brain [4]. The most commonly described antibodies are those directed against the voltage gated potassium channel (VGKC)-complex and its subtypes, glutamic acid decarboxylase (GAD) and N-methyl-D-aspartate receptor (NMDAR) [5].

* Corresponding author at: Istanbul University, Istanbul Faculty of Medicine, Department of Neurology, Units of Child Neurology and Clinical Neurophysiology, 34390 Capa/Istanbul, Turkey.
E-mail address: pinartopaloglu2000@yahoo.com (P. Tekturk).

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Epileptic encephalopathies (EE) are mysterious electroclinical conditions where cognitive and neurological functions deteriorate due to enormous epileptic activity and are mostly seen in infancy and early childhood [6]. Various etiological factors (developmental brain anomalies, metabolic, genetic and acquired lesions) have been demonstrated in a minority of the patients, while disease mechanisms of EE remain unclear in most of the patients [6]. West syndrome (WS) with epileptic spasms usually responding to adrenocorticotrophic hormone (ACTH) treatment and its follower in age spectrum, Lennox-Gastaut syndrome (LGS) presenting with many types of drug-resistant seizures are among the most frequently observed EEs. Some of these patients may also show propensity to respond to other immune treatment options such as intravenous immunoglobulin (IVIg); thus they could be considered as ideal candidates for "autoimmune epilepsy" [7]. Furthermore, WS patients have been shown to demonstrate dysfunction in cellular immunity, changes in Ig levels, and T lymphocyte dysfunction further supporting the autoimmunity hypothesis [8]. Moreover, in a recent case report with epileptic spasms, VGKC-complex antibodies were shown in association with status epilepticus [9]. There are currently no reports on the systematic screening of a cryptogenic EE cohort for the presence of various NAA except patients with acute onset febrile infection related epilepsy syndrome [10].

Possible new treatment modalities at least in a subgroup of EEs are of utmost importance for these patients. In this study, we aimed to investigate the frequency of the NAAs in a cohort of patients diagnosed with EE of unknown origin and to identify their clinical characteristics.

2. Methods

2.1. Material/Subjects

We included 50 consecutive patients, (female/male: 18/32; mean age: 10.84 (±8.89); range: 1–36 years; mean duration of epilepsy 9.34 ± 8.9 years, range 1–35 years) who were followed in Istanbul Faculty of Medicine, Department of Child Neurology unit between the years of 2012 and 2014 and had been diagnosed as EE with their typical clinical and EEG findings according to the ILAE criteria [11]. EE was defined as the cognitive, sensory or motor deterioration due to prominent epileptic activity as suggested and patients were grouped as WS, LGS and other/undetermined EE. Age and gender-matched 40 healthy volunteers were also enrolled as the control group. For each investigated antibody, sera of 4–5 autoimmune encephalitis or paraneoplastic syndrome patients that were previously found seropositive were used as positive control. Data regarding demographics such as age, gender, neurological symptoms, age at onset, epilepsy duration, seizure types, presence of febrile seizures, medical and family history, history of autoimmune disorders, medication at the time of serum sampling, response to treatment and detailed EEG and neuro-imaging findings were collected from the files. Their prognosis was categorized according to the Gross Motor Function Classification System (GMFCS), Manual Ability Classification System (MACS) and Communication Function Classification System (CFCS9) [12]. According to these classification system scores, patients were divided into four groups as good (normal motor and mental status or mild mental retardation), moderate (moderate motor and mental retardation), bad (severe motor and mental retardation) and exitus. To assess the cognitive status, Denver or Alexander tests were used depending on the age of the subjects. Response to immunotherapy was also divided into three groups as good response (≥50% seizure decline when compared to basal monthly seizure frequency), moderate (20–50% seizure decline) and no response (<20% seizure decline). Drug-resistant epilepsy was defined according to the ILAE commission proposal [13].

All patients underwent a detailed neurological evaluation with clinical examination, seizure history and routine EEG with scalp electrodes (32 channels, non-invasive EEG monitoring with 10–20 system electrodes and ECG electrodes). EEG investigations were reviewed by the first two authors to ensure the correct diagnoses. Background activity, epileptiform interictal and ictal patterns and specific EEG patterns such as extreme delta brush were also listed according to a standardized form. Moreover, all patients underwent magnetic resonance imaging (MRI) examinations with 1.5 T scanners with a standard epilepsy protocol and were evaluated by an experienced neuroradiology team. Seizures and syndromes were diagnosed according to the revised terminology, and concepts for organization of seizures and epilepsies of the International League Against Epilepsy (ILAE) Commission on Classification and Terminology [11]. The syndrome diagnosis of EE of unknown cause was supported by the EEG and neuropsychological tests, besides evaluation of biochemistry and MRI to identify symptomatic causes.

In an effort to exclude symptomatic causes of EE, brain MRI was supplemented with a detailed routine investigation scheme, including investigations of biotidinase deficiency, pyridoxine deficiency, mitochondrial disorders, glycosylation defects, phenylketonuria and other aminoaciduria, TORCH infections and peroxisomal and lysosomal disorders for differential diagnosis. Patients with tuberous sclerosis were also excluded. Moreover known genetic causes of EE were investigated and found negative in all participants.

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2.2. Autoantibody testing

Sera for antibody analyses were collected during routine outpatient interviews. None of the patients were under immunotherapy during blood collection and there was a minimum of 4 months between the last immunotherapy session and collection of sera. Age and gender-matched 40 healthy individuals were also enrolled as the control group. Cerebrospinal fluid of the patients were not collected and analyzed due to ethical issues. The study was approved by the local ethics committee. Informed consent was obtained from all parents/legal guardians before blood sampling. The sera of the patients and controls were kept at −80 °C until assayed and were investigated for serum antibodies against VGKC-complex antigens, LGI1, CASPR2, NMDAR, GLYR, GAD, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and type A gamma aminobutyric acid receptor ($GABA_AR$). Ion channel antibodies were investigated by binding to HEK293 cells transfected with plasmids containing the NR1/NR2 subunits of the NMDAR, GluR1/GluR2 subunits of the AMPAR, LGI1, CASPR2, α1 subunit of the GLYR and α1/γ2 subunits of the $GABA_AR$. Transfected cells were incubated with patients' sera (1:20) and the appropriate Alexa Fluor secondary antibody, as reported previously [14]. The binding intensities were scored visually on a range from 0 (negative) to 4 (very strong). Only scores ≥2 were accepted as positive to avoid nonspecific low positivity. For detection of antibodies to VGKC-complex antigens, a radioimmunoassay (RIA) kit (RSR, Cardiff, UK) was used. Finally, GAD antibodies were measured by ELISA, as indicated by the manufacturer's recommendations (Euroimmun, Luebeck, Germany).

In addition, antibodies to uncharacterized neuronal surface and synapse antigens were investigated as described previously. Briefly, sera (1:250) were incubated for 1 h at room temperature with neurons cultured from newborn (P1) rat hippocampus. After extensive washing, neurons were fixed with 3% formaldehyde for 15 min and incubated with Alexa Fluor 488-conjugated anti-human IgG (Invitrogen, UK) at 1:1000 for 45 min. Subsequently, the cells were permeabilized with 0.3% Triton X-100 in PBS for 15 min and were incubated for 1 h at room temperature with mouse monoclonal antibody to microtubule-associated protein 2 (MAP-2; 1:1000, Sigma-Aldrich, UK), a marker of axonal and dendritic processes. The cells were then immunolabeled with Alexa Fluor 548-conjugated anti-mouse IgG (Invitrogen) at 1:1000 dilution for 45 min. Cells were mounted and images were photographed under a Zeiss fluorescence microscope with a digital camera using the Zeiss Axiovision software.

Descriptive statistics were applied, and the two groups of patients with and without serum antibodies were compared with Fisher's exact test, chi-square test and independent samples *t*-test, where appropriate. SPSS 15 software (SPSS Inc, Chicago, IL, U.S.A.) was used and the significance level was set at $p < 0.05$.

3. Results

3.1. Study population

Mean age of the included patients was 10.84 ± 8.90 years (32 M, 18F) and the distribution of the diagnosed EE syndromes in these patients was as follows: 12 WS (8 M, 4F; mean age: 5.1 (±5.5)), 10 LGS (8 M, 2F; mean age: 17.2 (±11.9)), 10 WS + LGS (WS developed to LGS) (2 M, 8 F; mean age: 15.5 (±7.2)), and the remaining 18 EE patients were grouped as other EE-unspecified (14 M, 4F; 8.5 (±6.3)). Three patients with Ohtahara syndrome and one patient with Landau-Kleffner syndrome were also included in this latter group due to the low sample sizes (Fig. 1).

Mean follow-up period of these patients was 9.34 ± 8.90 years (median: 5.5; range: 1–35 years). All EEG recordings since the beginning of seizures were evaluated (mean number of EEGs per patient: 3.8 ± 2.07; median: 4). 48 patients (96%) had both sleep and awake recordings whereas 23 patients (46%) also had video-EEG monitoring studies. Most of them (80%) had drug-resistance according to the criteria. Serum samples were taken during the acute-active stage in 88% of the patients, whereas the remaining patients were in remission or under appropriate control with AEDs. Age of seizure onset differed from first day of life to 14 years

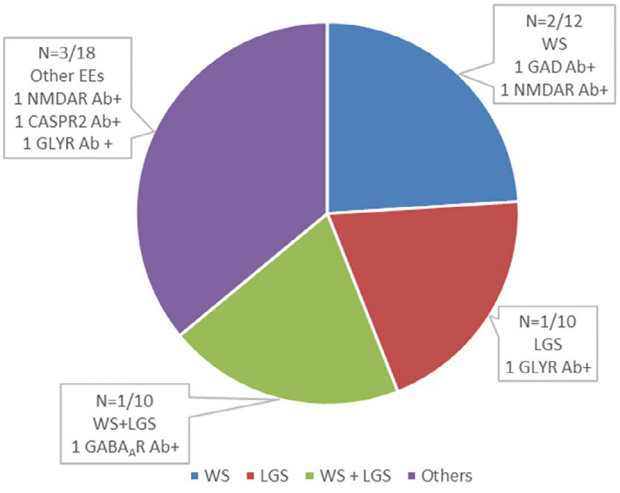

Fig. 1. Pie chart showing the distribution of the epileptic encephalopathy syndromes and associated neuronal autoantibodies. EE, epileptic encephalopathy; NMDAR, N-methyl-D-aspartate receptor; VGKC, voltage-gated potassium channel; CASPR2, contactin-associated protein-like 2; GAD, glutamic acid decarboxylase; GABAAR, gamma-aminobutyric acid type A receptor; GLYR, glycine receptor, WS: West syndrome, LGS: Lennox-Gastaut Syndrome, Ab: antibody.

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of age (mean: 22.54 ± 34.23 months; median: 9 months). Seizure frequency of the study group changed from 100/day to seizure freedom (mean: 5.8 ± 15.07; median: 1 per day).

Regarding the outcome, our study group showed 18% good, 40% moderate, 42% poor prognosis. Only one patient diagnosed with Ohtahara Syndrome died because of status epilepticus (2%).

3.2 Autoantibody results

Seven of the patients (14%) showed NAA positivity. Clinical characteristics and detected antibodies in these patients are shown in Fig. 1. We found two NMDAR antibody positive patients (4%) in the WS and other EE groups, respectively; one $GABA_AR$ antibody positive patient (2%) in the West + LGS group; one CASPR2 antibody positive (2%) patient in the other EE group; one GAD antibody positive patient (2%) in the WS and one each GLYR antibody positive patient (4%) in the other EE and LGS groups (Tables 1a and b). LGI1, VGKC-complex and AMPAR antibodies were not found in any EE patient. Moreover, none of the healthy controls were positive for any of the investigated auto-antibodies, whereas sera of previously diagnosed seropositive patients were all found positive for each individual antibody. EEG, seizure, anti-epileptic therapy characteristics, prognosis and immunotherapy responses of the seropositive group are summarized in Tables 1a and b.

Furthermore, sera of two patients who did not display any of the well-characterized ion channel and synapse antibodies, showed strong reactivity with the uncharacterized membrane antigens of cultured live hippocampal neurons (Fig. 2). One of the positive patients was a moderately motor and mentally retarded 25-year-old girl in the WS + LGS group. The other patient was a 10-year-old, severely motor and mentally retarded boy in the LGS group. Additionally, sera of seropositive patients 1, 2, 4 and 7 also showed reactivity with hippocampal neurons, whereas remaining EE and healthy control patients were negative for these hippocampal antibodies.

Clinical findings of seropositive versus seronegative patients are compared in Table 2. There were no differentiating EEG characteristics and extreme delta brush activity was not detected in our NMDAR antibody positive patients. Seventy-two percent of the study group had received immunotherapy (ACTH in all patients) before serum sampling. ACTH treatment was given for one and a half month by tapering slowly. IVIg treatment was given for five days with a total dose of 2 gr/kg. 75% of these showed good response while 19% and 5.5% showed moderate and poor response, respectively. 74% of the seronegative group had immunotherapy while four patients (57%) among the seropositive group received immunotherapy before serum sampling. Three of them (75%) showed good and one of them (25%) showed poor response. Good responders were one NMDAR antibody positive patient with WS, one $GABA_AR$ antibody positive patient with WS + LGS and one GAD antibody positive patient with WS while poor responder patient was NMDAR antibody positive patient belonging to other EE group.

After detection of NAA, seropositive patients were re-evaluated for further immunotherapy according to their seizure frequencies and neurological state. Three of the patients had good prognosis, one patient refused to be treated with IVIg and three patients had IVIg therapy (0.4 gr/kg for 5 consecutive days). IVIg responses of these three patients differed as good (GLYR antibody positive), moderate ($GABA_AR$ antibody positive) and poor (NMDAR antibody positive).

4. Discussion

This study has identified various NAAs in 14% of patients diagnosed with EE of unknown cause; a number which is comparable to other cohorts with epilepsy. Seropositive patients with EE did not show uniform and distinctive features and could not be differentiated from the seronegative group and thus routine antibody screening studies need to be done to all EE patients for detection of NAAs when the early immune treatment options are taken into account in this malignant group of epileptic syndromes.

Chou et al. reported 10 Taiwanese children with limbic encephalitis associated with antibodies against GAD among others [15]. VGKC-complex and NMDAR antibodies were also found in children with limbic encephalitis presenting with cognitive impairment and seizures [16]. It is thus tempting to speculate that subtle and unrecognized limbic encephalitis may underlie or mimic some atypical forms of EE. Remarkably three of our seropositive patients had atypical forms of EE, supporting this possibility.

Suleiman et al. described 13 representative children with suspected autoimmune epilepsy and proposed some guidelines for its recognition, such as evidence of CNS inflammation in CSF or on MRI, the presence of other autoimmune diseases and positive response to immunotherapy [3]. We hypothesized that EEs are candidates for autoimmune epilepsy due to their propensity to respond to immunotherapy. Interestingly, women were over-represented in the retrospective and selected cohort of Suleiman et al., as in other autoimmune disorders in general whereas our unselected cohort of EE had more seropositive male patients. The former study also reported that autoimmune epilepsy was associated with focal seizures more often and generally occurred in association with encephalopathy or other features of CNS dysfunction [3]. Although seizure characteristics of our

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Table 1a
Clinical characteristics of the neuronal auto-antibody positive patients with epileptic encephalopathy.

Patient number	Anti-neuronal antibody type, binding score[a]	Age[b]/sex	Type of EE	Age of onset	Family history/consanguinity	Previous infection/ inflammatory disease history	Neurologic examination	Cognitive impairment	MRI findings	Active stage during sampling	Outcome
1	NMDAR, 2	8/M	Other	4.5 years	No, consanguinity	No	Severe MMR, aphasia, serebellopyramidal syndrome, orofacial dyskinesia	Severe	Normal, then severe atrophic	Yes	Poor
2	NMDAR, 3	2/M	West	3.5 months	Mother AID, neutropenia/no	No	Moderate MMR	Moderate	Normal	Yes	Moderate
3	$GABA_AR$, 2	12/F	West + LGS	2 months	No, no	No	Severe MMR, spasticity	Severe	Normal	Yes	Poor
4	CASPR2, 2	6/M	Other	2.5 years	Epilepsy in his cousin, mother hyperthyroidism/no	Sepsis (two days old)	Head titubation, postural tremor	Mild	Normal	Yes	Good
5	GAD, (1200 U/ml)	4/M	West	9 months	Mother pernicious anemia, no	No	Mild hypotonia and then normal	None	Normal	No	Good
6	GLYR, 3	22/M	LGS	9 months	No, consanguinity	No	Moderate spasticity	Moderate	Normal (SPECT: left Frontal hypo-metabolism)	Yes	Moderate
7	GLYR, 3	8/F	Other	14 years	Epilepsy in uncle, consanguinity	No	Moderate MR	Moderate	Normal, then atrophic	Yes	Moderate

[a] Numbers indicate the antibody binding intensity scored visually on a range from 0 (negative) to 4 (very strong).
[b] Age during serum sampling for the current study of the patients.

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Table 1b
Characteristics related to epilepsy and EEG of seropositive patients investigated for neuronal auto-antibodies.

Patient no, (antibody)	Syndrome	Seizure types	Background EEG activity	Interictal epileptic activity	Ictal EEG	Treatment during serum sampling	AED response	Immunotherapy/ response	Other findings
1 (NMDAR)	Other EE	Myoclonia, myoclonic astatic, atypical absence, startle, gelastic, generalized tonic-clonic	diffuse theta and delta waves	Generalized, multifocal, spike-polyspike and slow waves, sometimes fast rhythmic activity with 2.5–3 Hz delta waves	Generalized polyspike and waves	LEV, VPA, LTG, ketogenic diet	Poor	ACTH – IVIg/ poor	Prominent photosensitivity on EEG
2 (NMDAR)	West	Infantile spasm	diffuse delta waves	Generalized hypsarrhythmia	Suppression burst pattern	VPA	Moderate	ACTH/good	
3 ($GABA_AR$)	West + LGS	Infantile spasm, myoclonia, tonic	diffuse theta and delta waves	First hypsarrhytmia and suppression burst pattern, then generalized multifocal spike and waves, rhythmic delta activity	Generalized tonic discharges with artefact	TPM, LEV, CLZ, VPA	Poor	ACTH (good) – IVIg (moderate)	Multiple ACTH cures and positive response to everyone
4 (CASPR2)	Other EE	Generalized complicated febrile seizures, atonic	diffuse theta waves to normal	Generalized, left frontal spike and polyspike and slow waves	Not available	LEV, VPA	Good	None used	Spiky K complexes during sleep
5 (GAD)	West	Infantile spasms, tonic, head drops	paroxysmal slow waves, otherwise normal	Generalized hypsarrhythmia, later bilateral frontocentrotemporal spikes	Spikes originating from left hemisphere	VPA then no drug	Good	ACTH / good	
6 (GLYR)	LGS	Myoclonic, myoclonic astatic, generalized tonic	diffuse theta and delta waves	Generalized multifocal spike and polyspike and slow waves	Generalized polyspikes	VPA, TPM, LEV	Poor	None used	Photosensitivity
7 (GLYR)	Other EE	Generalized tonic-clonic, generalized tonic	paroxysmal theta and delta waves	Generalized and sometimes focal spikes over right fronto-temporal region	Not available	CBZ, VPA	Moderate	IVIg/good	

ACTH: adrenocorticotropic hormone; AED, antiepileptic drug; AID: Autoimmune disease; CASPR2, contactin-associated protein-like 2; CBZ: carbamazepine; CLZ: clozapine; D, diabetes mellitus, EE: epileptic encephalopathy; EEG: electroencephalogram F, female; GABAAR, gamma-aminobutyric acid type A receptor; GAD, anti-glutamic acid decarboxylase; GLYR, glycine receptor, IVIg: Intravenous immunoglobulin; L: left R: right, LEV: levetiracetam; LGS: Lennox-Gastaut syndrome; LTG: lamotrigine; M, male; MMR motor-mental retardation MR: mental retardation MRI: magnetic resonance imaging, NMDAR, N-methyl-D-aspartate receptor; SPECT: single photon emission computerized tomography, TPM: topiramate; VGKC, voltage-gated potassium channel; VPA: valproic acid.

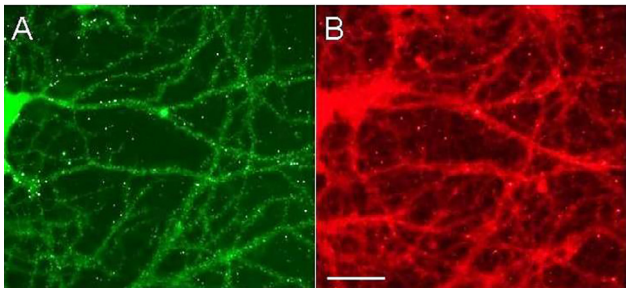

Fig. 2. Representative images of antibody assays performed using sera of patients with epileptic encephalopathy (EE). Cultured live hippocampal neurons incubated with the seronegative EE patient's serum demonstrate intense immunolabeling (green) of neuronal membrane and processes (A). Immunoreactivity of the EE patient's serum IgG significantly colocalize with that of microtubule-associated protein-2 (MAP-2) antibody (red) (B); Scale bar represents 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seropositive group showed generalized epileptic features especially among WS group, six of them (86%) also showed focal and multifocal characteristics as seen in Table 1b.

Previously reported NAAs associated with autoimmune encephalitis and seizures in children include NMDAR, VGKC-complex, GLYR, $GABA_AR$, $GABA_BR$, and GAD antibodies [4]. Our study supported these previous findings and added CASPR2 antibody to this spectrum. Although CASPR2 antibodies were previously thought to be found only in adults with Morvan syndrome and limbic encephalitis they were later shown to be positive in immunotherapy-responsive patients without typical features of limbic encephalitis [17]. A recent study revealed four CASPR2 antibody positive patients in a pediatric cohort of 178 patients that belonged to a group without immunotherapy history [18]. Our CASPR2 antibody positive patient had a good prognosis and since he was seizure-free with two antiepileptic drugs, he did not require immunotherapy. We have recently reported similar CASPR2 antibody positive patients with hippocampal sclerosis showing relatively favorable prognosis for this clinical constellation [19].

Moreover, association of EE with NAA was previously reported only in a case report before our cohort. A 4-month-old female infant of South Pacific origin with epileptic spasms and developmental delay was presented as the first case report with VGKC-complex antibodies [9].

Children have been noted to have seizures as the presenting symptom of NMDAR encephalitis more often than adults [20]. Seizures in NMDAR encephalitis are often of focal type, but can be secondarily or primarily generalized as well. Our two NMDAR antibody positive patients showed generalized seizures.

Antibodies against GAD are important players in the suggested guidelines for the identification of autoimmune epilepsy in children [3]. While some GAD antibody positive patients respond to immunotherapies, some are more resistant to immunotherapy than those with cell surface antibodies [21]. Our GAD antibody positive patient had classical hypsarrhythmia and diagnosed as WS. His remarkable difference from well-known patients with WS was his unexpected good

Table 2
Comparison of the clinical features of patients with and without neuronal autoantibodies.

	Seropositive patients N = 7	Seronegative patients N:43	p-values
Sex (female/male)	2/5	16/27	NS
Age at serum sampling (years ± SD)	Mean: 8.86 (±6.62) Median: 8 Range: 2–22	Mean: 11.16 (±9.23) Median: 10 Range: 1–36	NS
Epilepsy duration (years ± SD)	Mean: 7.14 ± 7.01 Median: 3 Range: 3–21	Mean: 9.70 ± 9.18 Median: 6 Range: 1–35	NS
Drug resistance	5	35	NS
Febrile seizures	1	2	NS
Comorbid disorders	1 mother with pernicious anemia, 1 mother with hyperthyroidism, 1 neutropenia	1 gluten enteropathy, 1 mother with SLE	NS
Mortality	1	0	NS
MRI abnormalities	4 cortical atrophy during follow up	16 mild atrophy during follow up (12 LGS, 2 West, 2 other EE)	NS
Status epilepticus	0	9	NS
Multifocal focus	4	28	NS
Generalized EEG features	7	33	NS
Fast rhythmic activity	2	19	NS
Good ACTH response	3	23	NS

AED: antiepileptic drug, SD: standard deviation. MRI: magnetic resonance imaging; ACTH: adrenocorticotropic hormone; N: number; SLE: systemic lupus erythematosus; NS: not significant.

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prognosis with a seizure freedom after first ACTH treatment.

Petit-Pedrol et al. recently reported antibodies against $GABA_AR$ in 18 of 140 patients (including seven children) with refractory seizures, status epilepticus, and encephalitis [22]. Our $GABA_AR$ antibody positive case was a severe motor and mentally retarded patient belonging to WS + LGS group who showed good response to ACTH during the early phase of her disease.

Hacohen et al. reported a 5-year-old girl with acute-onset EE and GLYR antibodies without further details [23]. GLYR antibodies were also reported in a child with refractory focal epilepsy, speech and behavioral disturbance and complete recovery after treatment with steroids [24]. Our two GLYR antibody positive patients corroborated the importance of these antibodies in EE and following immunotherapy, one of them showed a 50% decrease in seizure frequency per month with reported social improvement.

Presence of neuropil antibodies in further two patients without well-characterized antibodies suggest that there might be unknown cell surface antibodies pending to be discovered in EE patients.

Our study provided further evidence that specific autoimmunity may play a role in patients with various forms of EE. Potential clues for the possible role of autoimmunity in seropositive patients with EE were atypical prognosis of the classical EE type as observed in our GAD antibody positive patient with WS and atypical progression and neurological findings as observed in our NMDAR antibody positive patient with orofacial dyskinesia in addition to seizures.

Although our finding that NAAs were present in 14% of children with EE of unknown cause is very important, it is uncertain whether these autoantibodies are truly pathogenic. A potential study to prove pathogenicity of detected NAA would be to measure NAA levels before and after immunotherapy and show a decline in serum NAA levels in parallel to clinical amelioration. However, antibody positivity does not constitute an indication for advanced immunotherapy in chronic pediatric EE. Therefore, administration of immunotherapy in our retrospective study would raise ethical issues especially in a pediatric population.

Another approach would be passive transfer of serum IgG of seropositive patients to animals to induce clinical features of EE. Passive transfer of NMDAR and GAD antibodies have induced clinical features mimicking those of autoimmune encephalitis in previous studies [1,2]. Moreover, in vitro studies conducted with GlyR antibodies have induced complement activation and reduction of GlyR expression in cultured cells [3]. Currently, it is not certain whether NAAs of autoimmune encephalitis and chronic EE are capable of binding the same epitopes and exerting similar clinical features in experimental animals. Thus, pathogenicity of serum NAA of seropositive EE patients should further be studied in experimental animal models.

A disputable clue for pathogenicity of detected antibodies was presence of orofacial dyskinesia, a common finding of NMDAR encephalitis, in the NMDAR antibody positive patient 1. Also, increased muscle tonus observed in $GABA_AR$ antibody positive patient 3 and GlyR antibody positive patient 6 might conceivably be due to antibody-mediated suppression of the actions of target inhibitory neurotransmitter receptors. However, all these associations are rather speculative and should be further investigated in future studies [25–27].

Limitations of our study were that our EE cohort was moderate-sized and most of the patients were included years (mean: $7{,}14 \pm 7{,}01$; median: 3) after the start of the EE. On the other hand, children with EE who harbor NAA have not been studied before and our study is important for filling this gap.

In conclusion, NAAs were present in 14% of children with EE of unknown cause many years after the onset and may define a treatable subgroup of these childhood onset malignant epilepsy syndromes. Early and aggressive immune therapy might improve the clinical outcome in these patients. NAA positivity may be a good indicator of response to immunotherapy. So, NAA testing may be helpful during the decision of immunotherapy of EE patients showing potential clues for the possible role of autoimmunity such as atypical prognosis, atypical progression and neurological findings such as dyskinesia. Further prospective clinical treatment studies comparing the immunotherapy results of seronegative and seropositive patients with NAA titers are warranted.

5. Conflicts of interest

AV and the University of Oxford hold a patent for LGI1 and CASPR2 antibody tests, licensed to Euroimmun AG; AV receives a proportion of royalties. None of the other authors has any conflict of interest to disclose.

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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