

# The expanded clinical spectrum of anti-GABA<sub>B</sub>R encephalitis and added value of KCTD16 autoantibodies

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In this study we report the clinical features of 32 patients with gamma aminobutyric acid B receptor (GABA<sub>B</sub>R) antibodies, identify additional autoantibodies in patients with anti-GABA<sub>B</sub>R encephalitis that mark the presence of an underlying small cell lung carcinoma and optimize laboratory methods for the detection of GABA<sub>B</sub>R antibodies. Patients ( $n = 3225$ ) were tested for the presence of GABA<sub>B</sub>R antibodies using cell-based assay, immunohistochemistry and live hippocampal neurons. Clinical data were obtained retrospectively. Potassium channel tetramerization domain-containing (KCTD)16 antibodies were identified by immunoprecipitation, mass spectrometry analysis and cell-based assays. KCTD16 antibodies were identified in 23/32 patients with anti-GABA<sub>B</sub>R encephalitis, and in 1/26 patients with small cell lung carcinoma and Hu antibodies, but not in 329 healthy subjects and disease controls. Of the anti-GABA<sub>B</sub>R encephalitis patients that were screened sufficiently, 18/19 (95%) patients with KCTD16 antibodies had a tumour versus 3/9 (33%) anti-GABA<sub>B</sub>R encephalitis patients without KCTD16 antibodies ( $P = 0.001$ ). In most cases this was a small cell lung carcinoma. Patients had cognitive or behavioural changes (97%) and prominent seizures (90%). Thirteen patients developed a refractory status epilepticus with intensive care unit admittance (42%). Strikingly, 4/32 patients had a rapidly progressive dementia. The addition of KCTD16 to the GABA<sub>B</sub>R cell-based assay improved sensitivity of the in-house fixed cell-based assay, without loss of specificity. Twenty-two of 26 patients improved (partially) to immunotherapy or chemotherapy. Anti-GABA<sub>B</sub>R encephalitis is a limbic encephalitis with prominent, severe seizures, but patients can also present with rapidly progressive dementia. The co-occurrence of KCTD16 antibodies points towards a paraneoplastic origin. The addition of KCTD16 improves the sensitivity of the cell-based assay.

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**Abbreviations:** CBA = cell-based assay; GABA<sub>B</sub>R = gamma aminobutyric acid B receptor; KCTD16 = potassium channel tetramerization domain 16; mRS = modified Rankin scale; SCLC = small cell lung carcinoma; VGCC = voltage-gated calcium channels

## Introduction

Autoimmune encephalitis is a group of severe neurological disorders, some of which are associated with pathogenic autoantibodies directed at neuronal membrane proteins (Graus *et al.*, 2016), including the metabotropic gamma aminobutyric acid B receptor (GABA<sub>B</sub>R) (Lancaster *et al.*, 2010). The majority of patients with anti-GABA<sub>B</sub>R encephalitis present with limbic encephalitis with prominent seizures. Around 50% of the patients have an underlying small cell lung carcinoma (SCLC). Nearly all patients respond, completely or partially, to immunotherapy or a combination of immunotherapy and tumour treatment (Lancaster *et al.*, 2010; Hoftberger *et al.*, 2013; Jeffery *et al.*, 2013; Dogan Onugoren *et al.*, 2015; Chen *et al.*, 2017), stressing the importance of early diagnosis and treatment.

Diagnostic laboratories currently test the presence of GABA<sub>B</sub>R antibodies in serum or CSF with a (commercial or in-house) fixed cell-based assay (CBA) in which both GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits are expressed. Sensitivity of an in-house developed fixed CBA was reported to be ~100% for CSF and ~67–80% for serum (Hoftberger *et al.*, 2013). Alternatively, a live CBA, in which the surface of living transfected cells is stained with patient antibodies, can be used (own observation). However, live CBAs cannot be stored for later use and can therefore not be commercialized for widespread use. In some studies, additional immunohistochemistry of rat brain or immunocytochemistry of live hippocampal neurons in culture is used for confirmation of fixed CBA (Gresa-Arribas *et al.*, 2014).

In this study we: (i) provide a detailed description of the clinical and laboratory findings of 32 patients with anti-GABA<sub>B</sub>R encephalitis and show that besides limbic encephalitis anti-GABA<sub>B</sub>R encephalitis can also present with a rapidly progressive dementia; (ii) present a novel autoantibody directed at the intracellular GABA<sub>B</sub>R accessory protein potassium channel tetramerization domain containing 16 (KCTD16), which points towards a paraneoplastic origin; and (iii) evaluate the different laboratory methods that are available for the detection of GABA<sub>B</sub>R antibodies and show that the in-house fixed GABA<sub>B</sub>R-CBA can be improved by the addition of KCTD16.

## Materials and methods

### Patient inclusion

Samples from patients ( $n = 3225$ ) clinically suspected to have immune-mediated encephalitis were tested prospectively (May 2011 to Aug 2018) by routine diagnostic testing with immunohistochemistry and commercial CBA. Two hundred and eighty-two samples, collected for diagnostic testing of onconeuronal antibodies prior to the identification of GABA<sub>B</sub>R as an autoantigen (2000–2010), were tested retrospectively with immunohistochemistry and in-house fixed CBA. Lastly, in a cohort of 384 patients with clinical suspicion of Creutzfeldt-Jakob disease, 22 patients were retrospectively diagnosed with autoimmune encephalitis by a neuropathologist (Maat *et al.*, 2015). These 22 CSF samples were subsequently tested with immunohistochemistry and in-house fixed CBA. All diagnostic tests were carried out by the Erasmus MC University Medical Center (Rotterdam, The Netherlands), the Dutch national referral centre for paraneoplastic neurological syndromes and autoimmune encephalitis. This study was approved by the institutional review board and informed consent was obtained from patients or their relatives.

The control subjects ( $n = 329$ ) included plasma or serum from 46 anonymous healthy blood bank donors, 13 rheumatoid factor positive patients, 50 patients with SCLC without neurological symptoms (13 with limited disease, 31 with extensive disease, six with unknown disease grading) (Titulaer *et al.*, 2009), 26 patients with Hu syndrome and SCLC, 21 patients with Lambert-Eaton myasthenic syndrome (LEMS), voltage gated calcium channels (VGCC) antibodies and SCLC, 50 patients with amyotrophic lateral sclerosis (Huisman *et al.*, 2015) and 123 patients clinically suspected of autoimmune encephalitis.

### Clinical description

Clinical information was obtained retrospectively from medical records and telephone interviews with patients, relatives or treating physicians. Reduced consciousness was included as a symptom if not caused by a status epilepticus or induced by medication. We included the results of the first MRI, EEG and CSF examination carried out after disease onset. The number of antiepileptic drugs includes all medication known to control seizures that were administered (according to clinical letters), including intravenous drugs during intensive care unit (ICU) admittance. Seizure types were classified according to the International League Against Epilepsy Seizure Classification,

2016 (Fisher *et al.*, 2017). Severity of clinical symptoms were scored according to the modified Rankin scale (mRS) (van Swieten *et al.*, 1988). Treatment response was defined as a decrease of at least one point in mRS after immunotherapy. Patients were classified as having limbic encephalitis when the criteria were met as described in Graus *et al.* (2016). Rapidly progressive dementia was scored using the NINCDS-ADRDA classification (McKhann *et al.*, 2011). Dementia criteria needed to be met within 6 months after the appearance of the first cognitive symptom or the patient had died within 2 years after the appearance of the first cognitive symptom.

## Statistical analysis

Incidence rate was calculated with 95% confidence intervals (CI), using the number of patients identified prospectively in 2015–2017, assuming a Poisson distribution, using available Dutch population data (statline.cbs.nl/statweb/). Statistical analysis was performed using IBM SPSS Statistics 21 and GraphPad Prism 6.0. The following statistical tests were used when appropriate: Fisher's exact test, Fisher-Freeman-Halton test, Mann-Whitney U-test, and Wilcoxon signed rank test. Because of the exploratory nature of the study, *P*-values between 0.01 and 0.05 should be considered with caution.

## Laboratory procedures for diagnostic tests

Immunohistochemistry was carried out as previously described (Ances *et al.*, 2005). Briefly, rat brains were fixed with paraformaldehyde (PFA), cryoprotected, snap frozen and cut into sagittal sections. Sections were incubated with patients' serum (1:200) or CSF (1:2). The staining was visualized with diaminobenzidine and slides were counterstained with haematoxylin. Antigen retrieval using sodium citrate (pH 6) of paraffin embedded SCLC tissue samples was carried out prior to staining with rabbit anti-KCTD16 (1:200) (Sigma Aldrich). Neuronal cultures and staining were performed essentially as previously described (Kaech and Banker, 2006; Hughes *et al.*, 2010). In short, living hippocampal neurons of at least 14 days *in vitro* were incubated with patients' serum (1:50) or CSF (1:2) and were subsequently fixed and stained with a fluorescently-labelled secondary antibody.

Commercial CBA (Euroimmun) was used according to the manufacturer's recommendations. In short, human embryonic kidney (HEK) cells were co-transfected with unlabelled GABA<sub>B1</sub> and GABA<sub>B2</sub> and stained with patient serum (1:10) or CSF (undiluted). For in-house CBAs HEK cells were transfected with GFP-GABA<sub>B1</sub> (kind gift from Dr Lily Jan, UCSF, San Francisco) and GABA<sub>B2</sub> (RN214644, OriGene) with or without co-transfection of FLAG-KCTD16, KCTD12 or KCTD8 (kind gift from Dr Martin Gassmann, University of Basel, Basel) and were stained with patient serum (1:40) or CSF (1:2). The presence of KCTD antibodies was determined by fixed CBA with HEK cells transfected with the individual KCTD subunits. Titrations were performed using serial dilutions on fixed CBA. CBAs of serum and CSF, with and without co-transfection of KCTD16, were stained and scored in the same batch. For live CBA incubation with the patient sample (serum 1:40, CSF 1:2) was performed in culturing medium

prior to fixation. In addition, the samples were tested by the diagnostic immunology laboratory at Erasmus MC University Medical Center for the presence of a panel of classic paraneoplastic antibodies (anti-Hu, Yo, Ri, Ma1, Ma2, Tr, amphiphysin, VGCC and CV2) and anti-neuronal surface antibodies (anti-NMDAR, AMPAR, GABA<sub>B</sub>R, LGI1 and Caspr2).

## Mass spectrometry

Immunoprecipitation and mass spectrometry analysis were carried out as described previously (de Graaff *et al.*, 2012; van Coevorden-Hameete *et al.*, 2015). In short, protein was extracted from adult rat brains and incubated overnight with 10 µl of serum. After 16h, protA/G Sepharose beads (GE Healthcare Life Sciences) were added. The beads were washed, boiled and supernatant was loaded on a 4–12% Bis-Tris gel (Invitrogen) and sent for mass spectrometry analysis.

## Microscopy

Immunohistochemistries were scored visually on an Olympus BX50F. CBAs and live hippocampal neurons were scored visually by two independent observers using a Nikon eclipse 80i upright microscope. Confocal images were acquired with a Zeiss LSM 700 using the 40× and 63× (oil) objectives. Images were processed using ImageJ.

## Data availability

Any data not published within this article are available at the Erasmus MC University Medical Center. Patient-related data will be shared upon request from any qualified investigator, maintaining anonymization of the individual patients.

## Results

### KCTD16 antibodies are associated with an underlying SCLC

Thirty-two patients with anti-GABA<sub>B</sub>R encephalitis were identified, of whom 18 were diagnosed prospectively and nine retrospectively. In the five remaining patients, immunohistochemistry showed neuropil staining and live neurons showed surface labelling, but in-house GABA<sub>B</sub>R-CBA was initially scored negative (before optimization of the assays). In those patients antibodies to the GABA<sub>B</sub>R were detected using immunoprecipitation and mass spectrometry analysis. Next to the GABA<sub>B</sub>R subunits GABA<sub>B1</sub> and GABA<sub>B2</sub>, which confirmed the presence of GABA<sub>B</sub>R antibodies in these samples, in four of five patients the intracellular GABA<sub>B</sub>R-accessory protein KCTD8, KCTD12 or KCTD16 were pulled down (Supplementary Table 1). The presence of KCTD8, KCTD12 or KCTD16 was confirmed with KCTD-only fixed CBAs (Supplementary Table 2 and Supplementary Fig. 1). A subgroup of 23/32 (72%) anti-GABA<sub>B</sub>R encephalitis patients had KCTD16 antibodies. These antibodies were found in 1

of 26 (4%) patients with SCLC and anti-Hu syndrome, whereas 329 healthy and other disease control samples (including 50 patients with SCLC without PNS, and 21 patients with SCLC, LEMS and VGCC antibodies) tested negative. Anti-GABA<sub>B</sub>R encephalitis patients with KCTD16 antibodies had an underlying tumour more frequently. Of 28 patients who underwent sufficient tumour screening (either CT thorax and abdomen or FDG-PET-CT) (Titulaer *et al.*, 2011b), 18 of 19 patients with KCTD16 and three of nine patients without KCTD16 antibodies had an underlying tumour ( $P = 0.001$ ) (Fig. 1A). Patients with KCTD16 antibodies had significantly higher anti-GABA<sub>B</sub>R titres in CSF compared to patients without KCTD16 antibodies ( $P = 0.01$ ) (Fig. 1B), and tended to have a status epilepticus more frequently for which admission to the ICU was required ( $P = 0.045$ ) (Fig. 1C). No other factors (anti-GABA<sub>B</sub> titres in serum, maximum mRS during disease and response to immuno- and/or chemotherapy) differed significantly between patients with or without KCTD16 antibodies (Fig. 1D and E). SCLC biopsy tissue from a patient with KCTD16 antibodies expressed KCTD16, whereas normal lung tissue (Fig. 1F) and SCLC tissue from a patient without KCTD16 antibodies did not (data not shown).

## Patients and clinical phenotype

The characteristics of all patients are summarized in Table 1. The detailed clinical information for individual patients can be found in Supplementary Tables 3 and 4. Sixteen patients were male (50%). The median age at disease onset was 66 years. Incidence of anti-GABA<sub>B</sub>R encephalitis (calculated from January 2015 to December 2017) was 0.26/1 000 000 inhabitants/year (95% CI 0.14–0.44).

Limbic encephalitis was the main clinical syndrome in most patients (27/32; 84%). Of the five remaining patients one only had seizures and four (13%) had a rapidly progressive dementia. All four rapidly progressive dementia patients presented with a subacute cognitive decline and hallucinations/psychosis (Table 2). Two patients had myoclonia and/or cerebellar/pyramidal disturbance of movement. Creutzfeldt-Jakob disease was seriously considered in all four patients, and one patient fulfilled criteria for probable Creutzfeldt-Jakob disease, according to the CDC Diagnostic Criteria for Creutzfeldt-Jakob Disease, 2010 (<https://www.cdc.gov/prions/cjd/index.html>). Overall, most patients initially presented with seizures (53%), while the others had subacute cognitive decline or behavioural changes. None of these 15 patients were initially considered to have a primary psychiatric disorder. In all 15 patients there was a subacute onset of severe cognitive symptoms or behavioural disorders. Thirteen were directly referred to a neurologist (after visiting the emergency room or outpatient clinic), while two patients were first admitted to the department of internal medicine (one with hypertension and a cognitive disorder, and one with pneumonia and signs of delirium). During the disease course 31 of 32 patients

(97%) developed cognitive or behavioural problems. Nearly all patients (90%) experienced seizures. In all cases seizures were generalized, in 15% these were clear focal to bilateral tonic clonic seizures. In addition, eight patients experienced focal seizures, five of which with impaired awareness. In five cases the type of seizures was not described. Often the seizures were refractory to antiepileptic drugs. Thirteen patients (42%) developed a refractory status epilepticus for which admittance to the ICU was required. Additional symptoms that occurred frequently were psychosis/hallucinations (32%), language/speech problems (26%), reduced consciousness (23%) and headache/vomiting (19%). The median mRS was 4 [interquartile range (IQR): 3–5; range: 2–5] at maximum disease severity. There were no differences between non-tumour ( $n = 7$ ) and tumour patients ( $n = 21$ ) and specific clinical features [seizures presenting symptom, status epilepticus, maximum mRS (pretreatment), best mRS (post-treatment)], although the power was limited due to the sample size (Table 3).

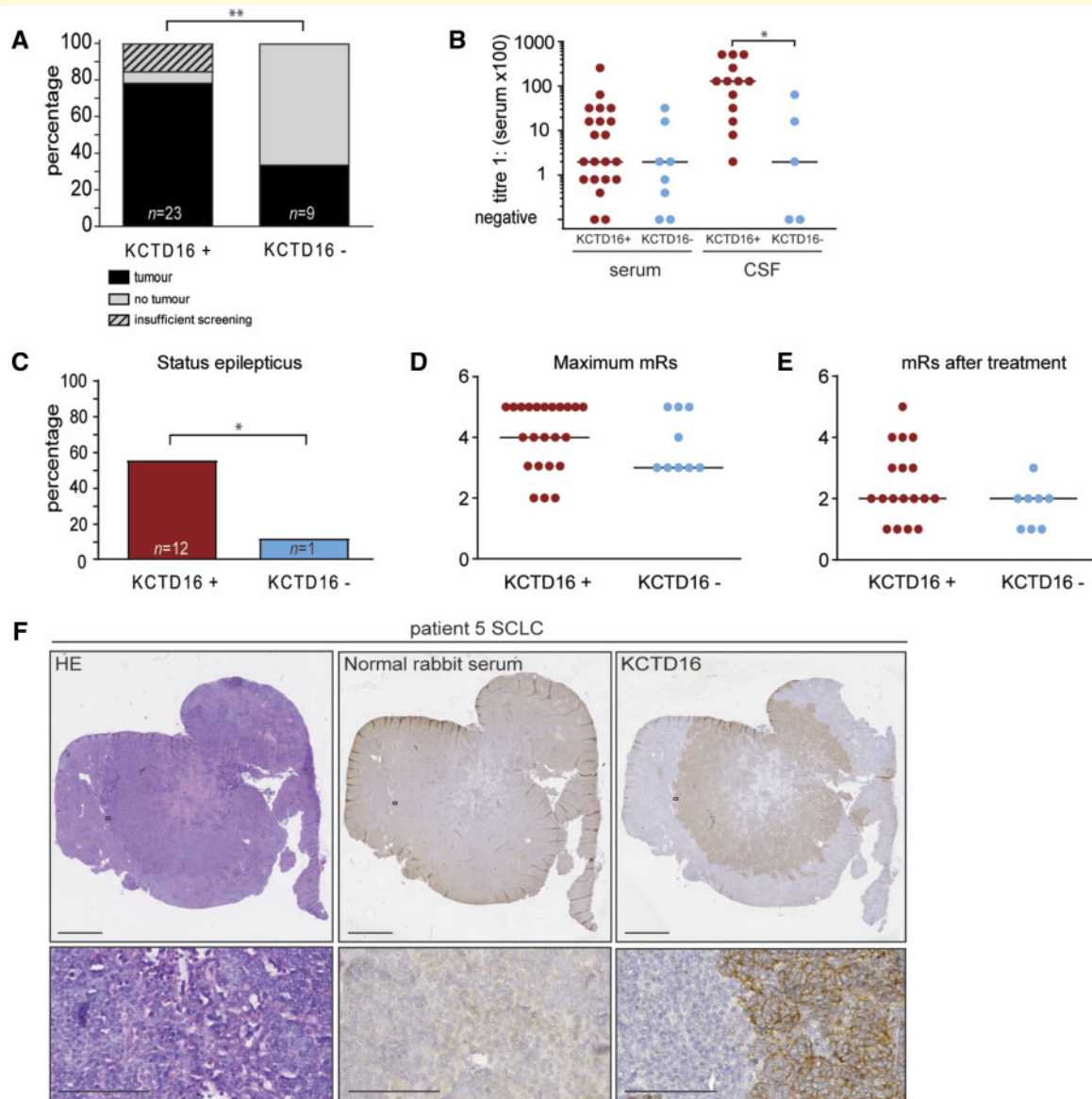
## Ancillary testing

The detailed results for ancillary testing from individual patients can be found in Supplementary Table 3. CSF analysis was carried out in 30 patients, and was abnormal in 29 (97%). The findings mainly included mild pleocytosis (76%) and increased protein level (36%). Increased IgG index and/or oligoclonal bands were reported in 9 of 11 patients, but were not determined in most patients. Initial MRIs were obtained in 29 patients and were abnormal in 45% of the cases, most frequently T<sub>2</sub>/FLAIR-hyperintensities of the mesiotemporal lobe (11/29; four unilateral and seven bilateral), one patient had atrophy of the mesiotemporal lobe and one patient had mesiotemporal hypointensity. Initial EEG results showed focal slowing (76%) often in combination with epileptic discharges (44%).

CSF analysis was available in three of four rapidly progressive dementia cases and showed a mild pleocytosis in all cases, often accompanied by oligoclonal bands or an elevated IgG index. In two of four patients, 14–3–3 was present in CSF and tau was very high (with relatively normal phospho-tau, ratio > 40). In the patient that lacked 14–3–3 protein in CSF, the EEG showed triphasic complexes typical of Creutzfeldt-Jakob disease. In the other cases the EEG was either normal or showed an aspecific encephalopathy. Together with the clinical findings one patient met the criteria for ‘probable Creutzfeldt-Jakob disease’.

In two of four patients with rapidly progressive dementia diagnosis of anti-GABA<sub>B</sub>R encephalitis was made post-mortem. In the remaining two, MRI and CSF abnormalities initiated the search for anti-neuronal autoantibodies and a possible underlying tumour, leading to the diagnosis of autoimmune encephalitis.





**Figure 1** KCTD16 antibodies are associated with an underlying tumour. **(A)** Bar diagram depicting percentages of patients with or without an underlying tumour. Patients with KCTD16 antibodies more frequently have an underlying tumour. Fisher exact test,  $P = 0.001$ . **(B)** Scatterplot depicting serum and CSF anti-GABA<sub>B</sub>R titres of patients with or without KCTD16 antibodies; lines indicate median values. GABA<sub>B</sub>R antibody titres in serum do not differ between patients with or without KCTD16 antibodies, whereas antibody titres in CSF are significantly higher in patients with KCTD16 antibodies. Mann-Whitney test,  $P = 0.24$  (serum),  $P = 0.01$  (CSF). **(C)** Bar diagram depicting percentages of patients with a status epilepticus. Status epilepticus tended to occur more frequently in patients with KCTD16 antibodies when compared to patients without KCTD16 antibodies. Fisher exact test,  $P = 0.045$  ( $P$ -values between 0.01 and 0.05 should be considered with caution). **(D)** Scatterplot depicting mRS at disease maximum, lines indicate median values. Maximum disease severity does not differ between patients with or without KCTD16 antibodies. Mann-Whitney test,  $P = 0.59$ . **(E)** Scatterplot depicting minimal mRS after treatment, lines indicate median values. Response to treatment does not differ between patients with or without KCTD16 antibodies. Mann-Whitney test,  $P = 0.20$ . **(F)** Immunohistochemistry of SCLC tissue from Patient 5, stained with haematoxylin and eosin (HE), normal rabbit serum and KCTD16 antibody. The image shows specific KCTD16 expression in tumour cells, which is absent in healthy lung tissue. Staining was performed on sequential slides and images were taken in the same area of the sample. Scale bars = 25  $\mu$ m.

## Tumour association and response to treatment

Tumour screening with (FDG-PET) CT of thorax and abdomen was carried out in 28 patients, of whom 16 (57%)

had an underlying SCLC, one patient had a small cell carcinoma of the bladder, and four (14%) had disseminated disease of a primary tumour of unknown type (in those patients no material for pathological examination could be obtained). Besides GABA<sub>B</sub>R antibodies, two patients

**Table 1 Patient characteristics (n = 32)**

Male: female	16: 16
Median age of onset (IQR, range)	66 (57–75, 44–85)
<b>Presenting symptom</b>	
Seizures	17 (53%)
Cognitive/(behavioural) <sup>a</sup>	6 (19%)
Behavioural/(cognitive)	9 (28%)
<b>Clinical syndrome</b>	
Limbic encephalitis	27 (84%)
Rapidly progressive dementia	4 (13%)
Epilepsy	1 (3%)
<b>Symptoms (during disease course)</b>	
Cognitive and/or behavioural	31/32 (97%)
Seizures	29/32 (90%)
Generalized	26/26 (100%)
Focal to bilateral tonic clonic	4/26 (15%)
Focal with impaired awareness	5/26 (19%)
Focal	3/26 (12%)
Hallucinations	10/31 (32%)
Language/speech	8/31 (26%)
Reduced level of consciousness	7/31 (23%)
Headache and/or vomiting	6/31 (19%)
Autonomic dysregulation <sup>b</sup>	4/31 (13%)
Focal neurological symptoms <sup>c</sup>	4/31 (13%)
Sleep disturbance	3/31 (10%)
Movement disorder <sup>d</sup>	2/31 (6%)
Cerebellar symptoms	1/31 (3%)
<b>Super refractory status epilepticus</b>	13/31 (42%)
<b>Median number of AEDs (IQR, range)</b>	2 (1–4, 0–6)
<b>CSF (first performed, n = 30)</b>	
Pleocytosis (median, range cells/mm <sup>3</sup> )	23/30 (17, 7–195) (76%)
Elevated protein	9/25 (36%)
Normal	1/30 (3%)
<b>MRI (first performed, n = 29)</b>	
Mesiotemporal T <sub>2</sub> /FLAIR hyperintensities	11/29 (38%)
Bilateral	7/11 (64%)
Unilateral	4/11 (36%)
Mesiotemporal atrophy	1/29 (3%)
Mesiotemporal hypointensities	1/29 (3%)
Normal <sup>e</sup>	16/29 (55%)
<b>EEG (first performed, n = 25)</b>	
Epileptic and encephalopathic	11/25 (44%)
Encephalopathic	8/25 (32%)
Epileptic	2/25 (8%)
Normal	4/25 (16%)
<b>Tumour</b>	
SCLC <sup>f</sup>	16/32 (50%)
Small cell bladder tumour	1/32 (3%)
Tumour, unknown type	4/32 (13%)
No tumour, sufficient screening	7/32 (22%)
No tumour, insufficient screening	4/32 (13%)
Other onconeural antibodies <sup>g</sup>	8/32 (25%)
<b>Treatment</b>	
Immunotherapy	14/31 (45%)
Tumour therapy	2/31 (6%)
Immunotherapy + tumour therapy	11/31 (35%)
No treatment	4/31 (13%)
Response to treatment	22/26 (85%)

<sup>a</sup>Symptoms were scored as cognitive/(behavioural) if patients mainly had cognitive symptoms, or as behavioural/(cognitive) if patients mainly had behavioural problems.

<sup>b</sup>Bradypnoea, tachypnoea, asystolia, bradycardia.

with an unknown tumour type had other SCLC-associated antibodies (anti-AMPA and anti-VGCC). Median time to tumour diagnosis after first contact with a physician was 6 weeks (IQR: 2–9; range: 0–62). One patient was screened by a pulmonologist for suspected lung cancer prior to the onset of the neurological symptoms, the other patients were diagnosed after the onset of neurological symptoms (96%).

Treatment data were available in 31 patients. Patients were treated with a combination of immuno- and tumour therapy [11/31 (35%)], immunotherapy alone [14/31 (45%)], tumour therapy alone [2/31 (6%)] or remained untreated [4/31 (13%)]. The majority of the patients [22/26 (85%)] responded to treatment, with a median best mRS of 2 (IQR: 1–3; range: 1–5) after treatment. In 19/21 patients with seizures and treatment response, seizure freedom was reached with a median time to seizure freedom after immunotherapy of 6 days (IQR 0–22, range 0–239). In 17/21 patients with cognitive symptoms and treatment response, cognitive symptoms improved after immunotherapy, with a median of 35 days (IQR 10–104, range 10–265). Seizure freedom was achieved faster than cognitive improvement ( $n = 20$ ;  $P = 0.012$ ). In 20 patients both cognitive improvement and seizure freedom were assessed after immunotherapy. In 13 patients (65%) seizure freedom was reached earlier than cognitive improvement, while five patients (25%) first showed cognitive improvement. In two patients seizure freedom and cognitive improvement were reached simultaneously (10%).

Two patients who did not respond had a poor overall physical condition and died shortly after immunotherapy before effects were assessable. Four patients did not receive treatment because of poor overall condition or because their disease presented prior to discovery of autoimmune encephalitis; one of these showed some improvement spontaneously. Two patients relapsed after 4 and 6 months, respectively. No tumour was found at relapse either.

At last follow-up, 13 of 32 patients were still alive (median follow-up 16 months; IQR: 8–27; range 2–109). Median mRS at last follow-up was 2 (IQR: 2–3; range 0–4). Median survival was 17 months (95% CI 7.80–26.20), not different between patients with tumours (15 months, 95% CI 11.04–18.96), or without (no median number as  $> 50\%$  survived,  $P = 0.36$ ; Fig. 2). The two (of seven) deceased patients without a tumour had their anti-GABA<sub>B</sub>R encephalitis diagnosis made post-mortem. One patient (Patient 26) had a rapid progressive dementia, presumed Creutzfeldt-Jakob disease, and was not treated. He died due to neurological

<sup>c</sup>Paresis arm, facial paresis, apraxia, gait instability.

<sup>d</sup>Myoclonus (2×).

<sup>e</sup>Two of eight patients with initial normal MRI developed mesiotemporal T<sub>2</sub>/FLAIR hyperintensities later in the disease course, and one patient developed mesiotemporal atrophy later in the disease course (five remained normal).

<sup>f</sup>Fourteen of 15 tumour patients (93%) smoked or had a history of smoking (in six patients data were not available).

<sup>g</sup>Hu (2×), VGCC (2×), GABA<sub>A</sub>R, GAD, Sox1, Ri, AMPAR (2×).

AED = antiepileptic drug; pleocytosis  $\geq 5$  cells/mm<sup>3</sup>; elevated protein  $\geq 0.58$  g/l.

**Table 2 Patient characteristics of the four patients with rapidly progressive dementia**

Characteristic	Patient 24	Patient 26 <sup>a</sup>	Patient 28	Patient 31
Sex	Male <sup>b</sup>	Male <sup>c</sup>	Female <sup>d</sup>	Male <sup>e</sup>
Age at onset	56	77	85	72
Tumour	No	No	No	No screening
Presenting symptom	Behavioural (/cognitive)	Behavioural (/cognitive)	Behavioural (/cognitive)	Cognitive (/behavioural)
Symptoms during disease course	Subacute cognitive decline, complete loss of memory and recognition, apraxia and hallucinations, sleep disturbance	Hypertension, psychotic behaviour, cognitive decline in days followed by cerebellar ataxia and aphasia	Pneumonia, 2 weeks later confusion, visual hallucinations, psychotic behaviour, memory deficit	Acute psychosis, within days followed by cognitive decline, only later on in disease course a few seizures and myoclonus
CSF	105 WBC, elevated protein, elevated IgG index, OCB; 14-3-3 positive; tau 12880, phospho-tau 95	18 WBC, elevated IgG index, OCB; 14-3-3 negative, tau and phospho-tau normal	-	15 WBC; 14-3-3 positive, tau 2450, phospho-tau normal
MRI	Hyperintensity mesiotemporal (bilateral)	Atrophy mesiotemporal, vascular white matter lesions	Normal	Hyperintensity mesiotemporal (unilateral)
EEG	Encephalopathic	Triphasic periodic complexes and encephalopathic	-	Normal
Autopsy brain	-	Perivascular inflammatory infiltrates consisting of B and T cells. Local infiltration in the hippocampus and basal ganglia. No evidence for CJD	-	Perivascular lymphocyte infiltration and gliosis left hippocampus. No evidence for CJD
Maximum mRS	4	5	4	5
Immunotherapy	MP + IVIg + rituximab	-	MP + IVIg + rituximab + cyclophosphamide	MP
Best mRS after treatment	2	5	3	5
Treatment response	Responded to therapy	Not treated	Some response to immunotherapy	Not treated
Follow-up, months	7	1 <sup>f</sup>	3	4 <sup>f</sup>

<sup>a</sup>Fulfilled criteria for 'probable CJD', but pathology refuted this diagnosis.

<sup>b</sup>See vertical line symbol in Supplementary Tables 3–5.

<sup>c</sup>See filled diamond in Supplementary Tables 3–5.

<sup>d</sup>See filled hexagon in Supplementary Tables 3–5.

<sup>e</sup>See open square in Supplementary Tables 3–5.

<sup>f</sup>Deceased.

CJD = Creutzfeldt-Jacob disease; IVIg = intravenous immunoglobulin; MP = methylprednisolone; OCB = oligoclonal bands; WBC = white blood cells.

deterioration. The other patient (Patient 27) was treated with methylprednisolone and intravenous immunoglobulins for a possible autoimmune encephalitis, but had a severe syndrome with status epilepticus, autonomic dysfunction and respiratory failure caused by a pneumonia. ICU treatment was discontinued after 1 month because of inconclusive diagnosis, older age, and a history of cognitive impairment. None of the other patients without tumour died after this acute phase. Eight of 14 deceased patients with tumour died of tumour progression. In the other six patients with a tumour, the cause of death was not reported. Three of four patients with insufficient tumour screening had died, two due to infection, while in one patient the cause of death was not reported. In six patients no initial improvement occurred prior to death of which four were not treated with immunotherapy. Until deterioration leading to death, the patients responded (partially), resulting in a median mRS of 2 (IQR 2–4; range 1–5).

## Addition of KCTD16 to GABA<sub>B</sub>R-CBA improves detection

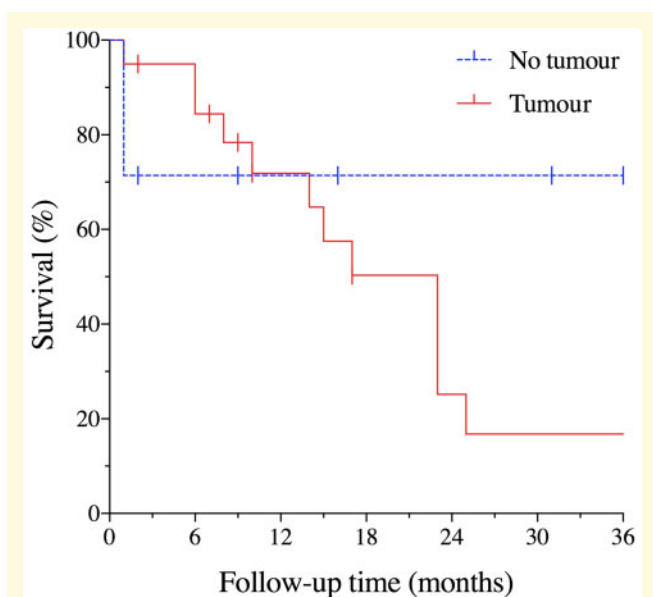
The patients' sera ( $n = 30$ ) and CSF ( $n = 21$ ) samples were tested for the presence of GABA<sub>B</sub>R antibodies using a set of different laboratory techniques (Fig. 3A–C and Supplementary Table 5). All sera and CSF samples showed neuropil staining on immunohistochemistry (Fig. 3A and D). All CSF samples and all but one sera tested, labelled the surface of live hippocampal neurons (Fig. 3B and D). Twenty-eight of 30 sera and 20/20 CSF samples were anti-GABA<sub>B</sub>R positive using live CBA (Fig. 3C and D). With commercial CBA GABA<sub>B</sub>R antibodies were detected in all but one serum (97%); however, the sensitivity of the CSF samples was 84% (16/19) (Fig. 3D). Three of 1125 serum samples tested in routine diagnostics were positive by commercial CBA without confirmation in CSF or by other laboratory tests and

**Table 3** Clinical features comparing tumour and non-tumour patients

	Tumour (n = 21)	No tumour (n = 7)	All (n = 28)	P-value
Seizures presenting symptom	13 (62%)	3 (43%)	16 (57%)	0.42
Status epilepticus	10 (50%) <sup>a</sup>	1 (14%)	11 (41%) <sup>a</sup>	0.18
Max mRS (pretreatment)	(n = 21)	(n = 7)		0.86
2	2	0	3	
3	7	2	9	
4	3	2	5	
5	8	3	11	
Best mRS (post-treatment)	(n = 19)	(n = 6)		1.00
1	5	2	7	
2	9	2	11	
3	3	1	4	
4	2	1	3	
Deceased at last follow-up	14 (67%)	2 (29%)	16 (57%)	
Survival in months, median (95% CI)	15 (11.04–18.96)	na (<50% deceased)	17 (7.80–26.20)	0.36

<sup>a</sup>For one patient, status epilepticus was unknown.

na = not applicable; see Fig. 2 for Kaplan-Meier curve. Median in this instance refers to the moment where 50% of patients had died; as <50% died, we are unable provide this value.



**Figure 2** Kaplan Meier curve of survival comparing tumour and non-tumour patients. Showing the data of patients with (n = 21) and without tumours (n = 7). Median survival was 17 months (95% CI 7.80–26.20), not different between patients with tumours (15 months, 95% CI 11.04–18.96), or without (no median number as >50% survived, P = 0.36).

considered clinically irrelevant, resulting in a specificity of 99.7%. In-house fixed CBA detected GABA<sub>B</sub>R antibodies in 26/30 (87%) of the sera and 18/20 (90%) of the CSF samples. When GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits were co-transfected with the GABA<sub>B</sub>R-accessory subunit KCTD16, the sensitivity for serum improved to 29/30 (97%) and in CSF to 20/20 (100%) (Fig. 3D). The addition of KCTD8 or KCTD12 to the CBA was inferior to KCTD16 (data not shown).

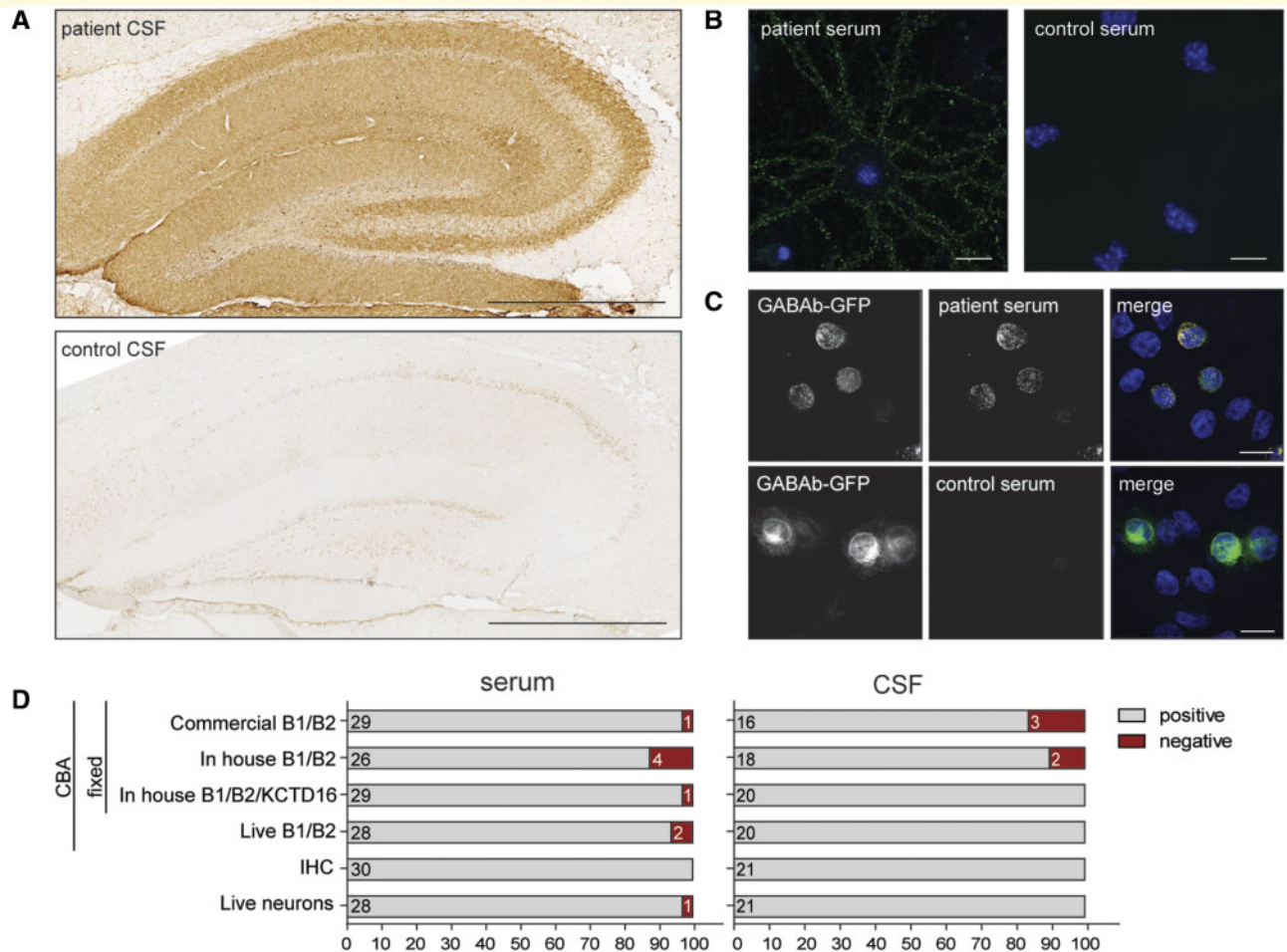
To validate the improvement of the fixed CBA by the addition of KCTD16, we performed serial dilution of all sera and 17 CSF samples on fixed CBA with and without co-expression of KCTD16. We observed a significant increase in titres detected with the CBA with KCTD16 co-expression (Fig. 4A–C). This effect was seen in both serum and CSF (serum 23/29, 79%, P = 0.00008; CSF 14/17, 82%, P = 0.001). Titres had a median 8-fold increase (IQR 2–52, range 0–800, P < 0.0001) in serum and a median 4-fold increase in CSF (IQR 2–7, range 0–256, P = 0.001). Fold changes in serum and CSF titres did not differ significantly between patients with and without KCTD16 antibodies. Also, the addition of KCTD16 to the fixed CBA did not result in a reduction of specificity as 193 healthy and diseased control samples tested negative. No differences were found between titres of patients with and without underlying tumours (Fig. 4D).

## Discussion

This study (i) identifies GABA<sub>B</sub>R antibodies in patients with rapidly progressive dementia in the absence of seizures, in addition to the majority of patients exhibiting limbic encephalitis with prominent and severe seizures; (ii) describes a novel autoantibody directed against the GABA<sub>B</sub>R accessory protein KCTD16, which is strongly associated with a SCLC; and (iii) shows that the addition of KCTD16 to the fixed GABA<sub>B</sub>R CBA results in improved detection of GABA<sub>B</sub>R antibodies.

Drug-resistant generalized seizures are the most prominent clinical feature of anti-GABA<sub>B</sub>R encephalitis. About half of the patients required ICU admittance to control seizures, which is more frequent than previously reported (Lancaster *et al.*, 2010; Hoftberger *et al.*, 2013). Moreover,



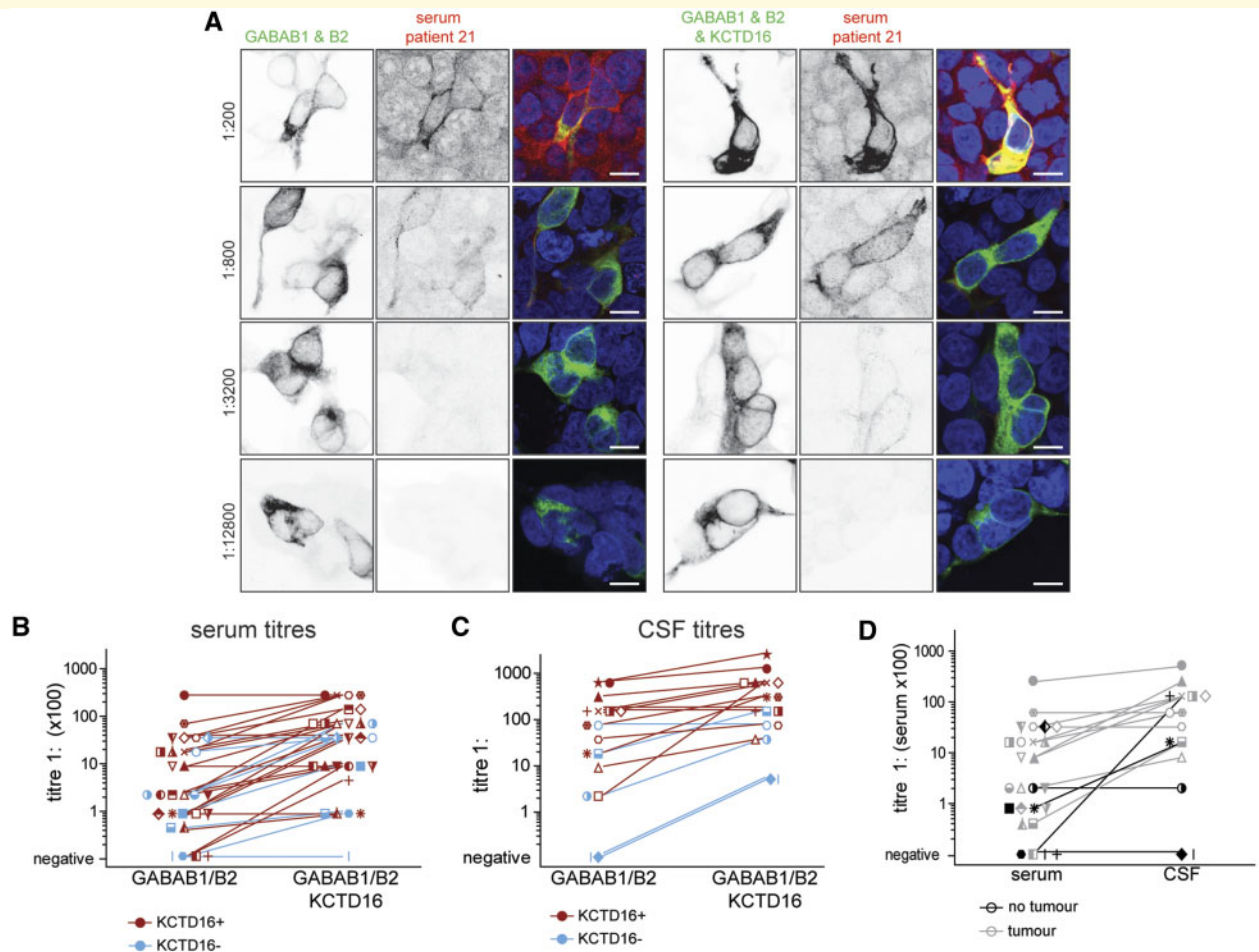


**Figure 3 Diagnostic tests for GABA<sub>B</sub> receptor antibodies.** (A) Immunohistochemistry of adult rat brain stained with patient CSF or control CSF. The patient CSF shows brain-wide neuropil staining, here exemplified by an image of the hippocampus. Scale bars = 500  $\mu$ m. (B) Immunocytochemistry of living rat hippocampal neurons. Labelling with the patient serum (green) results in a dot-like pattern along the neurites. Scale bars = 10  $\mu$ m. (C) Live in-house CBA of HEK cells transfected with GABA<sub>B1</sub>-GFP and GABA<sub>B2</sub> (green) and stained with patient serum or control serum (red). The patient serum labels the surface of cells transfected with GABA<sub>B</sub> receptor. Scale bars = 20  $\mu$ m. (D) Bar diagram representing the percentages of positive and negative tests for the different laboratory techniques that are used for the detection of GABA<sub>B</sub> receptor antibodies. For one patient, CSF was not available to perform CBA, but this sample did test positive for GABA<sub>B</sub> receptor in live CBA (Dalmau lab, Barcelona).

this number probably underestimates the occurrence of drug-resistant epilepsy, as only cases that required ICU admittance were taken into account. In contrast with anti-GABA<sub>B</sub>R limbic encephalitis with early and severe seizures, 4 of 32 patients presented with rapidly progressive dementia, of whom only one developed seizures late in the disease course. As literature on pure cognitive decline in patients with anti-neuronal autoantibodies is sparse (Geschwind *et al.*, 2008; Escudero *et al.*, 2017), many patients with rapidly progressive dementia are not investigated for antibodies. CSF abnormalities such as 14–3–3 protein, high phospho-tau/tau ratios did not discriminate between neurodegenerative disease and anti-GABA<sub>B</sub>R encephalitis. Clues for autoimmune encephalitis, such as (mild) pleocytosis, oligoclonal bands or typical MRI abnormalities might hint towards an autoimmune aetiology, and were helpful

in three of our cases. However, as ancillary testing might be normal and antibodies are not often thought of, patients with this treatable cause of dementia might not be diagnosed in general practice and denied treatment.

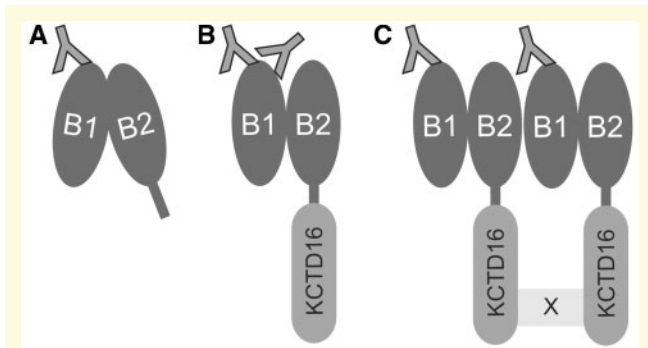
The presence of KCTD16 antibodies increases the probability of a paraneoplastic origin, and the necessity to screen more rapidly and more frequently. An underlying tumour occurred in almost all patients with KCTD16 antibodies, as opposed to three of nine patients without KCTD16 antibodies. Two paraneoplastic anti-GABA<sub>B</sub>R encephalitis patients lacked KCTD16 antibodies but had other SCLC-associated antibodies, anti-Hu and anti-VGCC, respectively. However, these were absent in the majority of the KCTD16-positive SCLC patients. Therefore, anti-KCTD16 seems to have additional value to other SCLC-associated autoantibodies, such as



**Figure 4** Endpoint titrations with fixed cell-based assay. (A) Titration of serum of an anti-KCTD16-negative patient (red) using a fixed CBA of HEK cells transfected with GABA<sub>B1</sub>-GFP and GABA<sub>B2</sub> (green) with or without co-transfection of KCTD16. Staining of cells co-transfected with KCTD16 can be detected up to a dilution of 1:3200, as opposed to without KCTD16 co-transfection, up to a dilution of 1:800. (B) Serum titres detected with a fixed CBA with or without co-transfection of KCTD16. Higher serum titres are detected with the addition of KCTD16 to the CBA. Median serum titre detected with the GABA<sub>B1/2</sub> assay was 200 (IQR 60–1600, range 0–25 600), and with addition of KCTD16 3200 (IQR 3200–9600, range 0–64 000;  $P < 0.0001$ ). (C) CSF titres detected with or without co-transfection of KCTD16. Higher CSF titres are detected with the addition of KCTD16 to the CBA. Median CSF titre detected with the GABA<sub>B1/2</sub> assay was 64 (IQR 7–160, range 0–512), and with addition of KCTD16 128 (IQR 48–512, range 4–2048;  $P = 0.001$ ). (D) Serum and CSF titres detected with a fixed CBA without KCTD16 co-transfection. Patients with an underlying tumour do not have higher titres in serum and CSF than patients without an underlying tumour. Mann-Whitney test, serum  $P = 0.23$ , CSF  $P = 0.41$ . Symbols in B–D refer to individual patients, which are explained in greater detail in Supplementary Tables 1 and 3–5.

anti-SOX1 (Sabater *et al.*, 2008; Hoftberger *et al.*, 2013), anti-Hu (Pignolet *et al.*, 2013) and anti-VGCC (Mason *et al.*, 1997), and can help in predicting a paraneoplastic origin of autoimmune encephalitis. The presence of KCTD16 antibodies in one patient with anti-Hu syndrome and SCLC shows the association of anti-KCTD16 with SCLC, also outside the context of anti-GABA<sub>B</sub>R encephalitis. However, we could not detect staining against KCTD16 proteins in the SCLC of one patient without paraneoplastic neurological syndrome, as has been published for Hu (Pignolet *et al.*, 2013) and SOX1 (Sabater *et al.*, 2008). It would be of additional value to study the expression of GABA<sub>B</sub>R and KCTD16 in more SCLC of patients without paraneoplastic neurological syndrome.

The presence of anti-KCTD16 is associated with higher GABA<sub>B</sub>R antibody titres in CSF and a more frequent severe status epilepticus. This is likely explained by the presence of an underlying tumour, which has also been associated with more severe disease in anti-NMDAR encephalitis (Gresa-Arribas *et al.*, 2014) and LEMS (Titulaer *et al.*, 2011a). Neither KCTD16, nor the presence of a tumour were predictive of survival. Although shorter survival time in SCLC-GABA<sub>B</sub>R patients is expected, this was not found. Likely explanations are the small number of idiopathic anti-GABA<sub>B</sub>R encephalitis patients, and the lack of a diagnosis before dying, leading to no or insufficient immunotherapy. The two deceased patients without tumour died within 2 months from symptom onset, had their diagnosis made



**Figure 5** GABA<sub>B</sub> receptor with and without KCTD16 co-expression. Schematic representation of the possible effect of the addition of KCTD16 to the CBA. (A) GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits expressed without co-expression of KCTD16. (B) GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits expressed with co-expression of KCTD16 resulting in a conformational change in the GABA<sub>B</sub> receptor that allows for more efficient antibody binding. (C) GABA<sub>B1</sub> and GABA<sub>B2</sub> subunits expressed with co-expression of KCTD16 resulting in clustering of GABA<sub>B</sub> receptors via the unknown scaffold protein 'X' resulting in more dense antibody labelling.

post-mortem and were offered no treatment or insufficient treatment. The other idiopathic anti-GABA<sub>B</sub>R encephalitis patients survived after the acute disease phase.

The fact that anti-KCTD16 co-occurs with SCLC suggests that their formation is a result of the aberrant expression of KCTDs, by SCLC tissue. Although most patients had both anti-KCTD and anti-GABA<sub>B</sub>R antibodies, suggesting aberrant expression of the GABA<sub>B</sub>R-KCTD complex, KCTD antibodies were not limited to anti-GABA<sub>B</sub>R encephalitis as one patient with anti-Hu syndrome and SCLC also had these. Unlike the GABA<sub>B</sub>R antibodies, the KCTD16 antibodies are directed at an intracellular antigen and most likely do not have pathogenic properties, although this was not tested within the scope of this study.

We show that co-expression of the intracellular auxiliary subunit KCTD16 with GABA<sub>B1</sub> and GABA<sub>B2</sub> in a fixed CBA improves the detection of GABA<sub>B</sub>R antibodies. Native GABA<sub>B</sub>Rs consist of two different core receptor units GABA<sub>B1a/b</sub> and GABA<sub>B2</sub>. These core receptor subunits control receptor surface expression, axonal and dendritic distribution, ligand binding and G-protein coupling (Gassmann and Bettler, 2012). In addition, the GABA<sub>B2</sub> subunit binds homo- or heterotetrameres of cytosolic auxiliary proteins belonging to the KCTD family (KCTD8, KCTD12, KCTD12b and KCTD16). The different KCTD family members show distinct expression profiles in the brain and bind to the intracellular part of GABA<sub>B2</sub> as a stable and obligatory part of the receptor at the cell surface. The KCTDs induce desensitization of K<sup>+</sup> currents in response to GABA<sub>B</sub>R activation in a subtype-specific manner (Schwenk *et al.*, 2010; Seddik *et al.*, 2012; Adelfinger *et al.*, 2014; Turecek *et al.*, 2014). Given the properties of KCTD proteins, there are several possible explanations for the improvement of the fixed CBA by the

addition of KCTD16 (Fig. 5); GABA<sub>B</sub>R antibodies are directed at a conformational epitope (Hoftberger *et al.*, 2013) and their binding might be suboptimal when the receptor is lacking an integral component, such as a KCTD protein (Fig. 5A and B) (Schwenk *et al.*, 2010). Alternatively, the co-expression of KCTD16 could lead to improved clustering of GABA<sub>B</sub>R on the cell surface, via binding of KCTD16 to a (currently unidentified) scaffold protein (Fig. 5C). A previous study shows that the detection of low-affinity antibodies to the acetylcholine receptor in myasthenia gravis can be improved by clustering the acetylcholine receptor in CBAs (Leite *et al.*, 2008). Lastly, the additional KCTD16 antibodies could be a partial explanation for the improved detection, as with the addition of KCTD16 the fixed CBA now also detects anti-KCTD16 titres. However, patients also lacking anti-KCTD16 showed increased titres with KCTD16 co-expression.

Importantly, the addition of KCTD16 to the fixed CBA increases sensitivity of the assay, without loss of specificity. Despite different optimization steps, some serum samples remained difficult to score by the in-house assay due to high noise levels. As the addition of KCTD16 increases the dilution the test can still be scored positive, the noise level becomes smaller. The net result is an improvement of the signal-to-noise ratio. With the addition of KCTD16 the fixed CBA performs as well as the live CBA with the advantage that it could be stored and is therefore suitable for use in many clinical diagnostic laboratories. For CSF samples the GABA<sub>B</sub>R-KCTD16 CBA performs better than the current commercial CBA. This diminishes the chance of a missed diagnosis if only CSF is sent for testing.

The main limitations of our study are because of its retrospective design and the low incidence of anti-GABA<sub>B</sub>R encephalitis when compared to anti-NMDAR encephalitis (Titulaer *et al.*, 2013) or anti-LGI1 encephalitis (van Sonderen *et al.*, 2016). This leads to the limited availability of clinical data and the lack of a standardized treatment regimen that could be evaluated. In addition, the retrospective identification of patients with anti-GABA<sub>B</sub>R encephalitis amongst samples collected for testing for onconeural antibodies could have led to biased results, as could be the case for the higher tumour frequency in our study when compared to previous studies (Lancaster *et al.*, 2010; Hoftberger *et al.*, 2013).

Overall, our findings have three major practical implications: (i) anti-GABA<sub>B</sub>R encephalitis should also be considered in patients with rapidly progressive dementia; (ii) KCTD16 antibodies can be used in clinical practice to determine the likelihood of an underlying SCLC in patients with paraneoplastic neurological syndrome and can lead to early tumour diagnosis and treatment; and (iii) the addition of KCTD16 to the fixed GABA<sub>B</sub>R CBA increases sensitivity without loss of specificity. Early diagnosis of anti-GABA<sub>B</sub>R encephalitis is of great importance, given the fact that most patients respond to treatment.



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## Competing interests

M.v.C.-H., M.d.B., E.d.G., D.B., M.S., J.D., M.R., E.H., M.N., S.B., J.H.V., J.V., C.H. and P.S.S. have nothing to report. M.T. has a patent pending entitled ‘Methods for typing neurological disorders and cancer, and devices for use therein’, which concerns the use of anti-GABA<sub>B</sub>R and anti-KCTD in diagnostic testing.

## Supplementary material

Supplementary material is available at *Brain* online.

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