UNIVERSITAT DE BARCELONA

Neurological syndromes associated with antibodies against the glutamic acid decarboxylase (GAD)

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DOCTORAL THESIS:

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A todos los que me animaron.
A Xabi, por su ayuda.
A mis padres, porque les debo (casi) todo.
A mi abuela, porque sí.

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CERTIFICAMOS que la siguiente tesis doctoral, presentada por Helena Ariño Rodríguez en formato compendio de artículos, ha estado realizada bajo nuestra dirección y consideramos que reúne las condiciones necesarias para ser defendida ante tribunal para optar al grado de Doctor en Medicina. No se prevé que las publicaciones que conforman el cuerpo de este trabajo sean incluidos en ninguna otra tesis.

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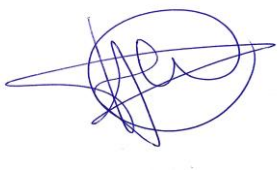

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Abbreviations

auto-HSCT	autologous hematopoietic stem cell transplantation,
CA	cerebellar ataxia,
CNS	central nervous system,
CSF	cerebro-spinal fluid,
GABA	gamma-aminobutyric acid,
GABARAP	GABA receptor-associated protein,
GAD	glutamic acid decarboxylase,
GAD-ab	antibodies against GAD,
GlyR-ab	antibodies against the glycine receptor,
IgG	Immunoglobulin type G,
IVIg	intravenous immunoglobulin,
LADA	latent autoimmune diabetes mellitus,
LE	limbic encephalitis,
MRI	magnetic resonance imaging,
mRS	modified rankin scale,
PERM	progressive encephalomyelitis with rigidity and myoclonus,
PET	positron emission tomography,
PLP	pyridoxal 5 phosphate,
PNS	paraneoplastic neurological syndrome,
RIA	radioimmunoassay,
SPS	stiff-person syndrome,
SPSD	stiff-person spectrum disorders,
T1DM	type 1 diabetes mellitus

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Introduction

Antibodies and neurological disorders

This thesis focuses on antibodies against the glutamic acid decarboxylase (GAD-ab) in neurological disorders. Antibodies (immunoglobulins) have multiple functions and can associate with autoimmune diseases when harboured against self-molecules. Antibodies recognizing neural antigens have been implicated in several neurological diseases, affecting either the central or the peripheral nervous system including the neuromuscular junction, but the significance of their detection may be very different. In some such diseases, autoantibodies are directly pathogenic, whereas in others they are indicative of an immune inflammatory process but may not directly cause the disease.

Pathogenic antibodies are implicated in antibody-mediated disorders. After binding its antigen, they cause the disease by reducing the surface expression, localization and/or functions of the antigen, or by recruiting other components of the immune system, such as cytotoxic natural killer cells and complement, resulting in tissue damage. Criteria to establish an antibody-mediated immunopathogenesis, based on the postulates of Witebsky[1], are the followings: 1) the direct demonstration of circulating antibodies; 2) the recognition of the specific antigen against which this antibody is directed; 3) the production of antibodies against the same antigen in experimental animals after immunization; 4) the appearance of pathological changes in the corresponding tissues of immunized experimental animal that are basically similar to those in the human disease. The best direct evidence of autoantibody pathogenicity can be obtained from passive transfer studies; an autoantibody is considered pathogenic if the recipient animals develop symptoms closely mimicking

those of the individuals with the autoimmune disease. Occasionally, direct evidence of autoantibody pathogenicity comes from pregnant women with autoimmune disease, in whom maternal antibodies may cause the same, although temporary, disease in the newborn after crossing the placenta. Examples of well characterized antibody-mediated disorders in neurology are myasthenia gravis with antibodies against acetylcholine receptor (AChR), which have shown to produce activation of the complement cascade or internalization of the receptors at the neuromuscular junction in rodents that develop the disease after passive transfer;[2] NMDA encephalitis with antibodies against the NMDA receptor, which have shown to produce internalization of the receptors at hippocampal neurons *in vitro* and in a rodent experimental model;[3,4] or neuromyelitis optica with antibodies against aquaporin-4 contained in astrocytes, which have shown to reproduce the pathological hallmarks of this demyelinating lesions when transferred along with complement.[5] In general, these antibodies are directed against cell-surface proteins, frequently synaptic proteins when CNS is involved, that are readily accessible to circulating autoantibodies from serum or CSF, and the reduction of antibody levels in patients correlates with the clinical course. To date, mainly autoantibodies of the IgG class have been shown to directly cause neurological disorders; the structure and subclass determine the effector mechanism.[6]

In other neurological disorders, antibody-associated disorders, the antibodies are not responsible for the onset of the autoimmune disease, but are surrogate markers of a disease. Their detection is the basis of several tests, so they are excellent diagnostic biomarkers. Although they are not able to trigger the disease themselves, it is likely that these antibodies can contribute to the disease by amplifying the autoimmune

reaction. The best example is the group of paraneoplastic neurological syndromes (PNS), the first CNS disorders that associated with neuronal autoantibodies.[7] The mechanism entails the ectopic expression by a tumour of an antigen that normally is expressed exclusively in the nervous system, and this antigen mounts a T-cell mediated immune response, aimed to control the growth of the cancer that ultimately causes the injury of the nervous system. In general, these antibodies target nuclear or cytoplasmic antigens that have been released subsequent to primary injury and efforts to decrease the antibody burden are less likely to cause a clinical improvement. Nevertheless, improvement of disease symptoms in response to immunotherapy is considered only circumstantial evidence of an autoimmune aetiology, like the epidemiological association with other autoimmune conditions or particular MHC haplotypes.

The issue related to GAD-ab in neurological diseases is that it is unclear what their role is. Are they pathogenic? Despite extended research, these antibodies are still difficult to classify. *In vitro* effects suggest that they could be pathogenic and the antigen that recognize is a synaptic protein relevant for neurotransmission, but there is no definite evidence that the antibodies can bind *in vivo* their antigen, which is intracellular, and the passive transfer of human antibodies do not reproduce successfully the disease in rodent models. Are they only biomarkers? In that case, for what? Do they have predictive or prognostic value? Whether their presence in a patient is predictive of outcome or treatment response, association with cancer and if the type of GAD antibody associates with specific clinical phenotypes are unsolved questions that we will try to address in this thesis.

Antibodies against GAD, biomarkers of different autoimmune diseases

GAD-ab were first identified in 1988 in the serum and CSF from one patient with stiff-person syndrome (SPS), type 1 diabetes mellitus (T1DM) and epilepsy.[8] Since its identification, GAD-ab have been detected in several autoimmune conditions affecting the endocrine system, the CNS or both simultaneously. This organ-specificity is because the glutamic acid decarboxylase is the enzyme responsible for the synthesis of gamma-aminobutyric acid (GABA) in neuroendocrine tissues, and is selectively expressed in GABAergic neurons and in the β-cells of the pancreatic islets.

GAD-ab are biomarkers of autoimmune diabetes and also have become excellent biomarkers of stiff-person syndrome (SPS), cerebellar ataxia (CA), epilepsy and limbic encephalitis (LE) of autoimmune origin. They have been exceptionally found in few cases of isolated nystagmus, palatal tremor, or brainstem dysfunction.[9] Frequently, GAD-ab are found in a more complex autoimmune background, since other organ-specific autoimmune diseases or biomarkers can coexist in patients with GAD-ab, including Hashimoto's thyroiditis, Grave's disease, pernicious anaemia, coeliac disease and vitiligo. GAD-ab are seldom detected in serum of healthy subjects (1%), individuals with other neurological conditions (5%),[10] and can appear along the clinical course in the juvenile form of neuronal ceroid lipofuscinosis Batten disease, result of mutations in the CLN3 gene.[11] Whether GAD-ab are pathogenic or whether all GAD-associated syndromes share the same pathogenic mechanism are still important questions despite recent advances.

GAD-ab can be demonstrated by several techniques, with different sensitivity depending on the characteristics of the antibodies. They can be

detected by immunoblots, using the denaturized protein, when the antibodies recognize antigenic determinants (epitopes) contained in a particular sequence of amino acids (linear epitopes). Immunoassays like immunoprecipitation or a cell-based assay using cells transfected with GAD, which maintain intact the tridimensional structure of the antigen, are appropriate to detect antibodies recognizing epitopes that are dependent on the three-dimensional structure of the antigen (conformational epitopes). GAD-ab can be detected also by immunohistochemistry using paraformaldehyde-fixed frozen rat tissues, which is a non-specific test for immunoreactivity against intracellular antigens with a sensitivity depending on the antibody level. Finally, there are tests able to detect the presence of GAD-ab and quantify at the same time the level of the antibodies. Two of these tests, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), have become the standard tests for GAD-ad detection in many laboratories because of these advantages along with the ability to detect low titres of antibodies. The combined use of these techniques showed from early investigations that GAD immune response differs between patients with endocrine and CNS disorders. The core differences in the GAD response between both groups of diseases are the titre of GAD-ab and their pattern of epitope recognition. Serum levels of GAD-ab are usually substantially higher (around 100 fold) in neurological disorders, and GAD-ab in T1DM are frequently unable to recognize linear epitopes. This could be relevant in the immune pathogenesis of both group diseases and could explain the fact that less than 5% of patients with T1DM will develop a neurological disorder associated with GAD.[12] One could argue that the relative immune-privilege of CNS due to BBB may justify this low prevalence of neurological disorder among patients with GAD-associated

T1DM. However, it does not explain the prevalence of T1DM among patients with neurological disorders; only around 1/3 of patients with SPS will develop concomitant T1DM at some point.[9]

GAD-ab in autoimmune diabetes

T1DM is the prototypic organ-specific autoimmune disease. It is the result of a selective immune-mediated destruction of the pancreatic β-cells, causing a chronic and irreversible deficiency of insulin. T1DM is strongly associated with haplotypes of HLA genes that are involved in antigen presentation. HLA haplotypes DR4-DQ8 and DR3-DQ2 alone or in combination may be present in nearly 90% of patients with T1DM diagnosed before 18 years of age.[13]

The pathogenic process begins years before the clinical onset. In the disease initiation, inflammatory infiltrates, mainly comprising dendritic cells, macrophages and B- and T-lymphocytes, invade the islets. This "insulitis" is thought to be initiated by CD4+ T-cells, which react to peptides of insulin. Once initiated by T-cells specific for insulin, islet cell damage ensues with concomitant release of autoantigens that results in the activation of an increasingly heterogeneous autoreactive T-cell repertoire. The mechanisms orchestrating the progression from non-invasive to invasive insulitis are not well understood, but it is likely that the highly proliferative nature of B-lymphocytes allows them to efficiently capture β-cell antigen for presentation to activated diabetogenic CD4+ T-cells and CD8+ T-cells, resulting in the rapid expansion of cells killing the islet β-cells.[14] At the time of clinical symptoms 60–80% of the β-cells are destroyed. Then, the development of autoantibodies to β-cell antigens is

likely a by-product of the autoimmune destruction of pancreatic cells. However, experimental work shows an active role of this autoantibodies in the pathogenesis of the disease promoting cytotoxic T cell responses.[15] In this sense, it is remarkable a phase II study of rituximab (a B-lymphocyte depleting monoclonal antibody) in patients newly diagnosed with T1DM significantly reduced the progression of insulin deficiency compared to placebo.[16]

GAD is one of the 3 major autoantigen in the islet cell autoimmunity. Approximately 70–80% of newly diagnosed T1DM patients harbour GAD-ab.[17] Autoantibodies to the β-cell (GAD, insulinoma-2-associated and the Zinc-transporter-8) can be found preceding the emergence of disease by up to several years. In individuals with a first-degree relative with T1DM, the presence of a single islet autoantibody only marginally increases the risk of T1DM. However, this risk increases with each additional islet auto-antibodies. Thus, the greatest value of the detection of GAD-ab is to be predictive markers to identify healthy subjects at high risk for T1DM, allowing the prompt identification of individuals who could benefit from the inclusion in prevention trials.[18]

Lately, screening of antibodies against islet antigens in adults, found that 5–10% of patients diagnosed with type 2 diabetes have GAD-ab and this predicts a risk for conversion to insulin dependence. This type of diabetes has also been referred to as latent autoimmune diabetes in the adult (LADA).[19] In this context, GAD-ab are important predictors of treatment response.

GAD-ab in neurological disorders

Only high serum GAD-ab levels (≥2000 U/ml by RIA) are demonstrated to have diagnostic value in neurological disorders.[9,20] However, a proportion of patients with T1DM (particularly frequent in the context of the autoimmune polyendocrine syndrome type II) might have high GAD-ab levels. Thus, an important feature in order to consider GAD-ab implicated in a particular neurological disorder is the presence of GAD-ab and oligoclonal IgG bands in CSF, which are present in more than 60% of patients with neurological disorders. The levels in CSF are lower than in serum, but proportionally higher than any other protein or IgG. The presence of oligoclonal bands that are specifically directed against GAD along with a high CSF GAD-ab index indicate a specific intrathecal synthesis of GAD antibodies in neurological disorders.[9,20–23]

The spectrum of GAD autoimmunity affecting CNS has expanded since the identification of GAD-ab in SPS. The majority of the research has been focused in this neurological disorder, and moved to other syndromes only in the recent years. Even though all the syndromes share the same autoimmune biomarker, it may well be that GAD-ab have a different role in each syndrome. Whether all GAD-associated syndromes share the same pathogenic mechanisms, or what renders certain brain regions vulnerable to autoantibody attack is not clear.

Stiff-person syndrome

SPS is a rare (estimated incidence of about one in a million) CNS disorder characterized by progressive muscular rigidity, predominantly of the trunk muscles, with superimposed painful spasms. Symptoms are frequently

triggered by unexpected stimuli, even light touch or emotional disturbance.[24] It has a female predominance and the age of initiation is variable including 5% cases presenting during childhood.[25] Muscle rigidity is caused by sustained muscular co-contractions concurrently occurring in agonists and antagonist muscles, which can be revealed by electrophysiological studies that characteristically show continuous activity of motor unit firing at rest.[26] This involuntary activity improves during sleep, anaesthetics or benzodiazepines, which is an important clinical criterion.

Diagnosis of this disorder is still based on clinical criteria. Since it first description in 1956 by Moersh and Woltman,[27] these criteria have been reviewed at least twice in 1967[28] by Gordon et al. and in 1989[24] by Lorish et al., which are the currently the accepted ones for the classic form of the disease: 1) Stiffness and rigidity in axial muscles (proximal limb muscles may also be sometimes involved); 2) abnormal axial posture (usually an exaggeration of the normal lumbar lordosis); 3) superimposed spasms precipitated by voluntary movement, emotional upsets and unexpected auditory and somaesthetic stimuli; 4) absence of brainstem, pyramidal, extrapyramidal and lower motor neuron signs, sphincter and sensory disturbance, and cognitive involvement (epilepsy may occur); 5) continuous motor unit activity (CMUA) in at least one axial muscle; 6) positive response to intravenous or oral diazepam (*minor criterion*)

Antibodies are not currently a diagnostic criteria, although some authors proposed the inclusion of GAD-ab as a minor criterion.[29] Indeed, they have demonstrated to be a very useful tool in daily practice to identify

some border-line cases that do not fulfil all major criteria (1-5 from the upper list).

Cases defined with the aforementioned criteria generally associates with the presence of GAD-ab (around 80%), have a relative good prognosis since remain ambulant for many years and respond to symptomatic drugs such as diazepam and baclofen.[30,31] However, many patients do not show the classic axial distribution of stiffness and spasms, or present additional neurological symptoms that exceeds the classic SPS. Marked progress has been made in the clinical and immunological characterisation of this disorder and allowed the recognition of SPS variants such as focal SPS (*stiff-limb*[32,33], or *stiff-trunk syndromes)*, jerking stiff syndrome, paraneoplastic variants or an infrequent severe presentation as encephalomyelitis (called progressive encephalomyelitis with rigidity and myoclonus or PERM).[34] And at the same time, investigations have shown that stiff-person disorder is a heterogeneous immunological condition that is associated with several antibodies other than GAD-ab. Pathogenic and clinical implications of this will be discussed later.

A common immunogenetic background has been noted for both T1DM and SPS due to overlapping alleles in the HLADRβ1 and DQβ1 loci in more than 75% of patients with T1DM and SPS.[35] Nevertheless, electrophysiological and pathological studies in SPS point to a selective dysfunction of certain brain circuits more than a cytotoxic damage, which is the hallmark in T1DM. The continuous activity of motor unit firing at rest that characterize these patients is attributable to the dysfunction of GABAergic inhibitory neuronal circuits, although it is not yet clear which circuits in the brain or in the spinal cord are primary involved.

Electrophysiological studies show a diffuse hyperexcitability of the motor cortex, as determined by paired pulse transcranial magnetic stimulation, but also a local loss of inhibition in the spinal circuits, as determined by H-reflex test using vibration-induced inhibition and reciprocal inhibition.[36,37] The fact that these electrophysiological parameters improve after immunotherapy and that are absent in patients with SPS without GAD-ab support a role of the immune-response in this GABAergic dysfunction.[37,38] Other kind of evidence of GABAergic dysfunction in patients with SPS is a significant decrease in GABA level in the sensorimotor cortex without structural changes measured by MRI spectroscopy,[39] a reduced postsynaptic $GABA_A$ receptor using a positron emission tomography (PET) study,[40] and a reduction of GABA levels measured directly in the CSF of patients.[20]

The findings in the few autopsies from patients diagnosed with SPS rarely show inflammatory infiltrates comparable to the insulitis seen in T1DM. Degeneration of anterior horn cells in the spinal cord with vacuolization, neuronal loss restricted to motoneurons and intense gliosis is the only finding in some cases.[41,42] In contrast, the rare variant PERM is a polioencephalomyelitis consisting in perivascular lymphocyte cuffing and neuronal loss essentially in the lower brainstem and spinal cord.[43–45] This pathological hallmark was the base to define PERM in the seminal descriptions.[46–48] In some other cases in the middle, clinically diagnosed with SPS, a mild perivascular lymphocytic cuffing in the brainstem and spinal cord has been found.[43–45] After a second look to the clinical description is interesting that all of them presented with atypical symptoms like respiratory arrest or cranial nerve involvement. Although some authors defend that PERM and SPS are different entities, these findings suggest a

clinic-pathological continuum from a functional disorder that may be partially rescued with symptomatic treatment, to a severe end of the spectrum with important inflammation extending beyond the spinal cord where the prognosis could be more dependent on the initiation of immunotherapy.

Prognosis of stiff-person syndrome disorders is generally unpredictable. Therapy is aimed at symptomatic relief, and modulation of the autoimmune process. Symptomatic treatment is based mainly on GABAergic drugs: central $GABA_A$ agonists like benzodiazepines, $GABA_B$ agonist like baclofen, or facilitators of GABAergic currents like other antiepileptic drugs. This symptomatic relief with drugs such as diazepam provides additional support to functional disruption of GABAergic circuits. This could be enough in initial stages or mild cases of SPS, but many patients need increasing dose reaching intolerable side effects that prevents high doses. Additional improvements occur among patients treated with immunotherapy, supporting the hypothesis that this disease is immune-mediated. The treatment seems to be associated with an ambulatory outcome in up to 70% of patients years after onset.[49] However, immunotherapy rarely causes the remission of the disease, which is a common feature for the GAD-associated neurological disorders. It is unknown the best therapeutic approach regarding immunotherapy. Nevertheless, SPS is the GAD-associated neurological disorder with a higher accumulated evidence. Controlled studies have been conducted only with intravenous immunoglobulin (IVIg) and rituximab. In a randomised-crossover trial, IVIg showed efficacy as compared to a placebo. A marked improvement in muscle spasms, mobility, frequency of falls and activities of daily living was observed in parallel with the reduction of GAD-ab titres and

the benefit lasted up to 1 year in some patients.[50] The trial with the B cell-depleting agent was not so promising, presumably because of strong placebo responses. Twenty-four patients were randomized to placebo or rituximab in a double-blind, placebo-controlled study. No significant treatment differences between the two groups were noted at 6 months. In spite of the overall negative study, 4 patients had definite improvement after this immunotherapy.[51] Interestingly, investigations in SPS showed that the humoral autoimmune response is composed of a rituximab-sensitive part that is rapidly cleared after treatment, and a rituximab-resistant component, presumably long-lived plasma cells.[52] Recently, a relevant publication reported 2 cases of severe SPS treated with autologous hematopoietic stem cell transplantation (auto-HSCT). Both patients achieved clinical remission with sustained and marked improvement in symptoms, and a return to premorbid functioning, more than 2.5 and 4.5 years after the procedure, despite the persistence of GAD-ab following auto-HSCT.[53] Thus, auto-HSCT emerges as a novel and promising therapy for these diseases and it success supports that clinical remission can be a goal.

Cerebellar ataxia

Years after the identification of GAD-ab, isolated descriptions[54–59] and 2 series of 14[60] and 17 (11 new)[9] patients, established the link between these antibodies and a late-onset cerebellar syndrome. The current prevalence of this disorder is not known. It is probably the second cause of autoimmune ataxia after paraneoplastic cerebellar degeneration and the prevalence can increase in the next years, since some cases attributed to other autoimmune causes are likely GAD-related.[61]

Among neurological disorders associated with GAD-ab, cerebellar ataxia is the second most frequent neurologic disorder after SPS.[9] Overall, there were no apparent differences between patients with cerebellar ataxia and SPS regarding GAD-ab titres, presence of intrathecal synthesis of GAD antibodies, and the presence of oligoclonal CSF bands.[9] The only particularity is that intrathecal GAD-ab production can be higher in patients with SPS and CA combined (2.5-fold higher comparing 5 to 33 typical SPS).[62] Similar to SPS, there was also a frequent coexistence of other organ-specific autoimmunities, with a higher prevalence of autoimmune DM than in general population (50% along the course of the neurological disease).[9] This syndrome presents a female predominance (more than 90% of patients are women) with a mean age of onset between 47-59 years old. The main cerebellar sign, which is present in all reported patients, is gait ataxia followed in frequency by limb ataxia and nystagmus in 65-85% of patients (depending on the series). A slow progression from presentation, suggesting a degenerative disease, is more frequent (around 60%) than a subacute onset mimicking a paraneoplastic cerebellar degeneration. However both presentations are possible.

Patients with cerebellar syndrome share the same biomarker, the same epidemiological and co-morbidity association than SPS with GAD-ab. Can we assume a similar pathomechanism and a similar clinical course? At the time of diagnosis, the most frequent situation is the absence of cerebellar atrophy. Moreover, there is evidence of dysfunction of the GABAergic system using functional neuroimaging in patients with cerebellar syndrome and GAD-ab in analogy to what is found in SPS.[63] A study performed in 3 patients using combined [11C]-flumazenil (a radiotracer that binds selectively to the $GABA_A$ receptor, especially in the cerebellum) and [18F]-

fluorodeoxyglucose (FDG) PET, showed a decreased flumazenil binding in the present patients. In 2/3 patients, this cerebellar GABAergic impairment was not associated to a decreased metabolic rate of glucose that could reflect neuronal loss. Nevertheless, neuroimaging reveal some kind of cerebellar atrophy in approximately 50% of patients after a variable follow-up time, indicating a special vulnerability to autoimmune injury in this brain area.[60]

There are scarce post-mortem studies, but they show specific neuronal death in patients with prolonged cerebellar ataxia and GAD-ab. In one patient that died from pneumonia after 5-year clinical course, the microscopic examination showed almost complete depletion of the Purkinje cells with Bergmann gliosis in absence of brain lymphocytic infiltration or macroscopic atrophy. At the same time, there were lymphocytic infiltrates in the pancreas and the selective decrease in the pancreatic islets corresponded with the pathological findings of autoimmune insulin-dependent diabetes mellitus.[64] This finding is similar to the post-mortem study of other female patient with cerebellar ataxia and GAD-ab who died from a terminal purulent meningitis.[65] Besides a lepto-meningitis, there was a selective loss of Purkinje cells with Bergmann gliosis and no perivascular lymphocytic cuffing or parenchymal infiltrates.

It is unknown which mechanisms ultimately cause the damage of the Purkinje cell. An indirect effect of GAD-ab through excitotoxicity by the selective suppression of inhibitory postsynaptic currents, or direct mechanism immune-mediated could be responsible. Be that as it may, it results in the selective death of the only efferent neuron in the cerebellum.

Hence, when that point is reached, the likelihood of functional recovery declines.

In contrast to SPS, GABAergic drugs in patients with cerebellar ataxia and GAD-ab are not associated with symptomatic improvement.[63] Therefore, immunotherapy is the only available treatment for them. While SPS with GAD-ab tends to be less disabling, cerebellar ataxia with GAD-ab can associate with a poor prognosis. However, the information about long-term outcomes is scarce and the benefit from treatment is unknown in patients with GAD-associated cerebellar ataxia. Publications of isolated cases report have shown contradictory effects with steroids, intravenous immunoglobulins, rituximab or azathioprine, all of them alone or combined.[55–57,66–69] One possible explanation is that the therapeutic window is narrow in patients with cerebellar ataxia. In a series of 62 GAD-ab-positive patients from the Mayo Clinic with a combination of cerebellar (63% of patients), brainstem, extrapyramidal, and spinal cord symptoms, the proportion of patients disabled by the disease was 60% in a median time of 30 months of disease duration.[70] In this retrospective study, the delay from symptom onset to initiation of treatment was significantly longer for patients who did not improve with immunotherapy.

Considering that cerebellar syndrome in the context of GAD autoimmunity may associate depletion of Purkinje cells, the delay in the initiation of immunotherapy could be crucial for the outcome of these patients. Currently, the factors associated with a better outcome are not well known. Therefore, o*ne of the main objectives of this thesis was to analyse the clinical course of this group of patients, and the long-term effect of the immunotherapy.*

Epilepsy and limbic encephalitis

Epilepsy is known to be a concomitant disorder (around 10%) in SPS with GAD-ab since its early description.[12] But this condition can be the sole manifestation of GAD autoimmunity. Patients with GAD-ab typically develop a longstanding pharmacoresistant temporal lobe epilepsy (TLE).[71] Although autoimmunity is a rare cause of epilepsy, the prevalence of GAD-ab may be around 20% in a subgroup of epileptic patients with TLE of adult onset, and absence of precipitant factors (history of viral encephalitis or meningitis, febrile seizures or head trauma).[72,73] The frequency in non-selected epileptic patients, though, is less than 5%.[74,75]

Patients with epilepsy and GAD-ab can initiate the disease with a limbic encephalitis, defined by a brain magnetic resonance imaging (MRI) revealing mediotemporal hyperintensity without atrophy in T2/FLAIR sequences and disturbance of episodic memory.[72] Isolated seizures may be the first manifestation of the disease and abnormal cognitive or psychiatric functioning is not prominent, so these patients can be easily misdiagnosed. Intrathecal synthesis of the specific antibody is present in all patients. Regarding the clinical course, there is evidence from a prospective study of 9 GAD-positive patients among a cohort of 53 patients with LE.[72] After a mean follow-up of 17 months in 7 patients, none of them were seizure-free and all of them received at least intravenous methylprednisolone pulses besides antiepileptic drugs.

Histopathological information is available from 9 patients who underwent surgery for incontrollable epilepsy.[72,76,77] The microscopic investigations revealed a characteristic neuropathological hippocampal sclerosis (HS), the ILAE type 3 HS or “end folium sclerosis” (CA4

predominant neuronal cell loss and gliosis), which is the least common form of HS based on a series of 135 surgical resections, and, clustered T lymphocytes in the hippocampus. The prominent chronic inflammation contrast with the post-mortem findings in SPS and CA. Furthermore, in one case was observed apposition of multiple GrB+ lymphocytes to single neurons, consistent with a specific cytotoxic T cell attack, but in a lower extent than in paraneoplastic encephalitis with onconeural antibodies.[76] Another piece of evidence pointing towards a more relevant role of T lymphocytes in GAD-related epilepsy is the study of a single patient with limbic encephalitis, and high GAD-ab levels, who was treated with a chimeric anti-interleukin-2 receptor monoclonal antibody (basiliximab). This monoclonal antibody blocks the receptor on the surface of antigen-activated T lymphocytes. In this patient, flow-cytometry analysis of CSF revealed an increased fraction of activated HLA-DR+CD8+ T-lymphocytes despite pulses of methylprednisolone. Consecutive analysis showed that the fraction of activated T-lymphocytes was normalized under basiliximab treatment and, later on, also the GAD-ab concentration was reduced. This immunological course was correlated with a temporary reduction of seizures.[78]

These findings open the door to a different immunopathogenesis in patients with limbic encephalitis and epilepsy, where the GAD-ab may be less relevant than in SPS or CA. Especially in those cases who evolve to secondary mesial sclerosis, which can be *per se* a potential epileptogenic trigger. Therapy in these cases with a dual mechanism cannot be extrapolated from the findings in cerebellar and SPS cohorts, and this challenging issue deserves specific research that has not been included in this thesis.

GAD as onconeuronal antigen

Patients with neurological syndromes associated with GAD-ab are not considered at risk for cancer, and extensive search for a tumour is not indicated unless they harbour additional onconeural antibodies. However, there are case reports of patients with GAD-ab whose cancer was identified by the time of the neurological diagnosis, suggesting a paraneoplastic mechanism.

The neurological syndromes in these few case reports are diverse, and also the kind of associated tumour. There are reports of limbic encephalitis, cerebellar ataxia, and encephalomyelitis associated with small-cell lung cancer (SCLC), non–SCLC, thymic carcinoma or pancreatic endocrine neoplasm.[9,79,80] Whether these cases represent a casual association or a true paraneoplastic trigger is unclear. A finding that support a true paraneoplastic origin is that, in one case in which it had been studied, the tumour expressed ectopically the antigen, and the sera from the patient immunoreacted with GAD-expressing tumoral cells.[79] In these case, GAD-autoimmunity is probably triggered by the hidden neoplasm. It is unknown whether the mounted immune response is different in any extent to the non-paraneoplastic cases. However, the post-mortem study of an isolated case showed a common final effect from an anatomo-pathological point of view compared to patients with no associated tumour. In this case, the brain autopsy of a patient that presented a paraneoplastic encephalomyelitis, showed an extensive loss of Purkinje cells in the cerebellum, proliferation of Bergmann astrocytes and vacuolization in the posterior columns of the spinal cord. Inflammatory infiltrates were not detected in the cerebellum or other areas of the nervous system.[79]

Nevertheless, the long-term outcome in cases associated to a hidden neoplasm is probably more dependent on the type of cancer that on the neurological condition and a prompt recognition may be essential. Therefore, an important question is *whether we are able to identify patients at risk of cancer when we detect GAD-ab associated with a neurological disorder.* In this thesis we collected all the cases identified in our laboratory with neurological disorders, GAD-ab, and absence of other onconeuronal antibodies to address this question.

Immunopathogenesis of neurological syndromes associated with antibodies against GAD. Are these antibodies pathogenic?

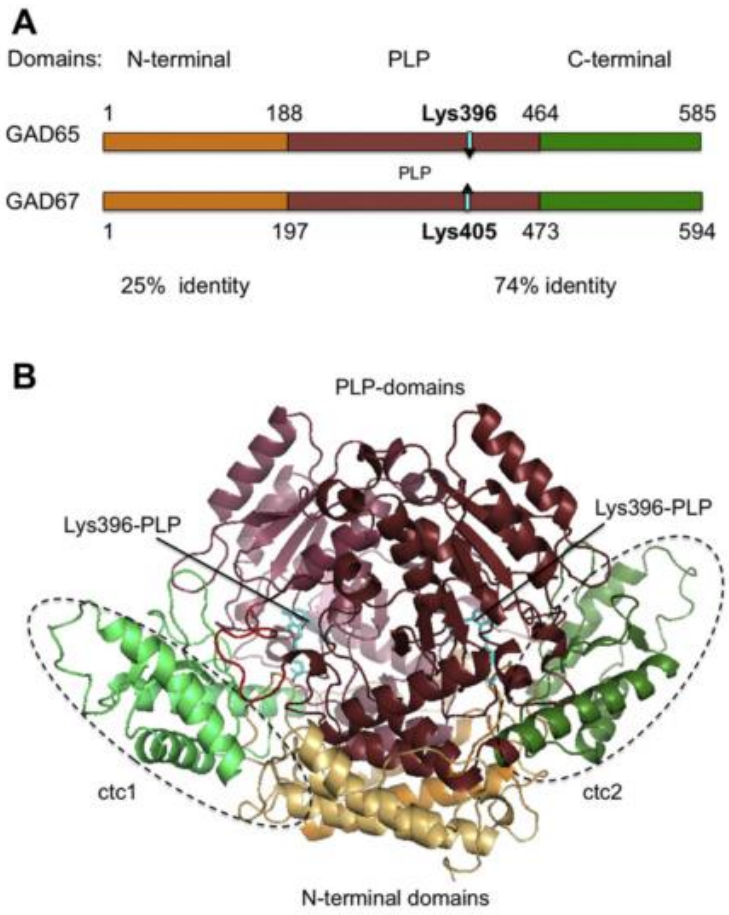
As has been previously discussed, a fundamental pathomechanism of the neurological dysfunction is the disturbance of GABAergic inhibitory neuronal circuits. Antibodies against GAD target the enzyme that is ultimately responsible for the synthesis of this neurotransmitter. Hence, the logical hypothesis is that these antibodies might be pathogenic. In fact, *in vitro* studies support this effect. Antibodies from patients with stiff-person syndrome or cerebellar ataxia cause the inhibition of GAD enzymatic activity reducing GABA production[81,82] and alter the GABAergic transmission affecting the presynaptic compartment in whole-cell patch-clamp studies using rat hippocampal or cerebellar slices[83,84]. This effect is dose dependent, however is not related to GAD-ab titres in patient's sample[82], and it can be reversible by removing the antibodies from the media or recovered by drugs which facilitate the release of GABA (e.g. forskolin, an activator of A kinase).[85]

The antigen

The glutamic acid decarboxylase is the rate-limiting enzyme that catalyses the decarboxylation of L-glutamate to gamma-aminobutyric acid (GABA) and is selectively expressed in GABAergic neurons as well as in pancreatic beta cells. The enzyme has two isoforms with distinct cell distribution and function: GAD65, a membrane-associated form enriched in the synaptic vesicles or pancreatic microvesicles, is responsible for the rapid synthesis of GABA; GAD67, a soluble form in the cytoplasm of the GABAergic neurons, is involved in functions such as synaptogenesis, but

not in neurotransmission. Thus, both isoforms are involved in the biosynthesis of GABA, the most abundant inhibitory neurotransmitter in CNS and they function synchronously to produce and regulate physiological levels of the neurotransmitter in different situations. GAD65 is seen as a regulatory enzyme that adapts the GABA level in situations of sudden demand and GAD67 might be involved in the synthesis of GABA for general metabolic activity.[86] GAD67-knockout mice die shortly after birth. In contrast, GAD65-knockout mice may suffer from epilepsy and stress-induced seizures but survive to adulthood.[87] Each of them is produced by two different genes, GAD2 and GAD1, respectively, but share 65% of the amino acid sequence. Both have 3 functional domains (the amino-terminal domain, the middle domain where the catalytic site resides, and the carboxy-terminal domain) and differ in their amino acid sequence, which contains the signals that anchor GAD65 in the synaptic vesicles. Both the GAD65 and GAD67 enzymes form obligate dimers (Fig1.) There is, however, a striking difference in the structure of the catalytic loop of the two isoforms,[88] which is consistent with a stable binding of the coenzyme pyridoxal 5 phosphate (PLP) to GAD67, whereas GAD65 oscillates between an inactive apoenzyme and an active holoenzyme. It has been postulated that the inherent flexibility of GAD65, not present in GAD67, may be the cause of an increased antigenicity in GAD65.[89] This may be relevant since the prevalence of antibodies against both isoforms is different, as will later be discussed.

Fig.1. Structural features of glutamic acid decarboxylase (GAD)



(A) Depiction of "traditional" three domains of GAD monomers, GAD65 and GAD67 and binding site in the PLP domain of the co-factor L- Pyridoxal 5 phosphate (PLP, cyan with black arrow). (B) Dimeric structure of GAD65 (amino acids 84-585) showing the structural locations of the three domains. The two C- terminal epitope clusters (ctc1 and ctc2) are indicated by dotted ovals.

Adapted from *Ali F, Rowley M, Jayakrishnan B, Teuber S, Gershwin ME, Mackay IR. Stiff-person syndrome (SPS) and anti-GAD-related CNS degenerations: protean additions to the autoimmune central neuropathies. J Autoimmun. 2011 Sep;37(2):79-87*.

However, although the antigen seems to be relevant, and *in vitro* evidence points to a potential pathogenic role of GAD-ab, several concerns arise from clinical observations regarding the pathogenic significance of these antibodies. First, in SPS patients there is no correlation between GAD-ab titres (in serum or CSF) and severity or duration of the disease.[21,90] Second, GAD-ab-positive neurological syndromes do not respond well to immunotherapy compared to those associated with antibodies against neuronal surface antigens like anti-NMDAR encephalitis. That means that, although patients may benefit from immunotherapies at some extent and achieve some degree of clinical improvement, the neurological syndrome is rarely fully recovered and it can easily become chronic.[50,51] But the most important issue is that GAD is a cytoplasmic antigen and it is unknown how antibodies can reach the intracellular compartment.

It has been proposed that GAD65 may be exposed to the outer side of the plasma membrane during the process of GABA exocytosis, since it is attached to the inner surface of the synaptic vesicles, but this has not been clearly demonstrated.[91] On the other hand, antibodies have been proved to penetrate living neurons.[92,93] Studies conducted in rats in the late eighties showed that IgG injected intraperitoneally were uptaken by motor neurons of the CNS.[92] Recently, another set of experiments demonstrated that anti-ganglioside antibodies are removed from circulation in mice by neuronal endocytosis and retrogradely transported to the motor neuron cell body in the spinal cord.[94] These neurons have the particularity that their axons project outside of the blood-brain barrier, in contrast to the rest of neurons in CNS. Another possible exception to neuronal exclusion of antibodies is

the Purkinje neuron, which is able to uptake *in vivo* different molecules including IgG.[95] Several experiments by the group of Dr. Greenlee show that paraneoplastic IgG recognizing intracellular antigens in Purkinje cells (anti-Yo, anti-Ri or anti-Yo antibodies) are taken up by viable neurons in slice cultures and show specific antibody-mediated neuronal death.[93,96,97] This may have implications in the pathophysiology of patients with cerebellar ataxia and GAD-ab. Penetrating live Purkinje cells has not been demonstrated for GAD-ab, but the internalization by viable neurons *in vitro* was demonstrated once by flow cytometry and indirect fluorescence using an immortalized rat mesencephalic cell line (AF5).[98] Despite these concerns about the intracellular nature of the antigen, there is an example of successful attempt to generate an animal model by passive transfer of autoantibodies against intracellular antigens, in particular against amphiphysin. This is a protein located in the pre-synaptic compartment of the GABAergic synapse involved in synaptic vesicle endocytosis and antibodies are found in a paraneoplastic form of SPS. The series of experiments using antibodies against amphiphysin showed that antibodies were able to bind *in vivo* its antigen and produce electrophysiological and clinical effects.[99,100]

In addition, two other pieces of evidence argue against a direct pathogenic role. First, GAD-ab do not transfer the disease from mothers to infants despite being present at high titres up to 24 months after birth.[101] However, even in the case of myasthenia gravis where the antibodies are well documented to be pathogenic, only 20% of infants develop myasthenia gravis. Second, autologous hematopoietic stem cell transplantation led to the resolution of clinical manifestations in 2 patients

with SPS despite the persistence of GAD-ab 2.5 and 4.5 years after the procedure, although this was only tested in serum.[53]

Experimental animal models to study pathogenicity of GAD-ab

A key step to confirm the direct pathogenic role of the antibodies in the development of neurological symptoms is to demonstrate such an effect *in vivo*. Several efforts have been made to induce disease in animals by passive transfer of human GAD-ab or, less frequently, by active immunization. So far, there are no convincing animal models. Only partial effects have been elicited, mainly electrophysiological abnormalities, and robust clinical or histological effects are generally absent.

The first study used local injections of purified IgG from patients with GAD-ab-associated SPS or cerebellar ataxia into anesthetized rats, and achieved clinical effects resembling in part the symptoms that present patients with SPS.[102] Thus, one dose at the lumbar paraspinal level in rats receiving GAD-IgG from patients with neurological disorders but not from T1DM patients induced repetitive muscle discharges, abnormal exteroceptive reflexes, and increased F-waves/M-response ratios after stimulation of the left tibial nerve, suggesting that these antibodies can enhance motoneuronal activity. Likewise, the intracerebellar injection inhibited the normal increase in the cortical motor response associated to repeated somatosensory stimulation only after infusion of GAD-IgG from neurological patients, which supports a dysfunction of the cerebellum in the normal modulation of this response. Furthermore, these investigators used microdialysis to study the effect on glutamate and nitric oxid production, and found an impaired synaptic regulation of glutamate

induced by the antibodies, supporting the possibility of excitotoxicity by increased concentrations of this neurotransmitter as a mechanism of cell death.[102]

Nevertheless, further studies were not able to reproduce consistently the results or achieve robust clinicopathologic correlations. In experiments conducted by the same group that demonstrated the pathogenicity of antibodies against amphiphyisin, intracerebral and intrathecal injections with purified IgG elicited some marginal aspects of the patients' symptomatology.[103,104] In this set of experiments, histological studies demonstrated a widespread deposition of the injected IgG in the brain of the treated animals, with predominant overlay of GABAergic neurons by double immunofluorescence. However, it is noteworthy that some relevant areas such as cerebellum or brainstem were not involved.

Another work used intracerebellar injections of purified IgG (from patients with SPS or cerebellar ataxia) or monoclonal GAD65-ab, and found a mild motor or cognitive effects such as decreasing the exploration behaviour or impairing the navigational strategies on the Morris Water Maze test.[98,105] A major concern in these experiments is the use of a monoclonal GAD65-ab (b78 and b96.11) obtained from the serum of a patient with high antibody titres, polyendocrinopathy but no neurological symptoms, so the interpretation of the findings is not straightforward.

All the aforementioned experiments were based on intrathecal or intracerebral injections. In a recent work, systemic injection of purified IgG from 2 patients with SPS and subsequent disruption of the blood-brain barrier with lipopolysaccharides did not achieve any behavioural effect.[106] The same negative result was obtained after active immunization

experiment with GAD65. Despite harbouring antibodies against GAD, this was not followed by any behavioural abnormality.[107] In both set of experiments, human and mouse IgG were demonstrated to bind some brain areas, but mostly hippocampus and circumventricular areas and furthermore, IgG were also found in the non-immunized mice. Interestingly, the binding of the patients' IgG or antibodies harboured by immunization with GAD65 was not completely abolished after adsorption with purified GAD protein and the remaining staining in cultured cerebellar neurons was interpreted as immunoreactivity against yet unknown antigens in the surface of neurons, suggesting the likelihood of pathogenic antibodies besides GAD-ab in neurological disorders associated to GAD autoimmunity.

The aforementioned concerns cast doubt on the pathogenicity of GAD-ab. In terms of causality, it seems that they are not sufficient *per se* to produce a neurological dysfunction. Although they recognize a relevant antigen and i*n vitro* studies suggest a direct effect, the passive transfer of human antibodies do not reproduce successfully the disease in rodent models. Then, the fourth criteria for an antibody-mediated disease is not satisfied and the search for other pathomechanisms is appropriate.

Alternative pathogenic hypothesis to GAD-ab in neurological disorders

T-cell studies

If GAD-ab are not enough to clearly reproduce clinic-electrophysiological effects, it is possible that GAD-related neurological disorders are a T-cell mediated disease. However, in the few available post-mortem studies in

humans, there are no findings to support this hypothesis unless in cases of PERM and to some extent in limbic encephalitis. T-cell infiltrates in cerebellum or spinal cord are generally absent despite neuronal degeneration and concomitant lymphocytic infiltration in the pancreatic islets.[64,108–110]

There are only few studies that have investigated GAD65-specific T-cell reactivity in SPS patients, in contrast to research in the field of T1DM and obtained different results. In one study using the sample from a patient with SPS, an increase in IFN-γ production was observed when peripheral blood lymphocytes were stimulated with GAD65[90], whereas other group found that only patients with CA in contrast to SPS or patients with endocrinopathies showed a significantly high production of IFN-γ after GAD stimulation.[113] In another study, GAD65-reactive T cell lines isolated from the peripheral blood of a patient with SPS had immunoregulatory features in proliferation assays and cytokine production profile, suggesting a GAD65-specific cellular autoimmunity suppression.[114] In experimental animals, it was shown that mice possessing a monoclonal GAD65-specific CD4+ T cell population developed a lethal encephalomyelitis disease in absence of any other T cells or B cells.[115] GAD65-reactive CD4+ T cells were found throughout the CNS in this model.

In view of these findings, a T-cell mediated cytotoxicity in GAD-associated neurological disorders is unlikely, but still T cells may have an important paper in mounting a specific immune-response. Generation of high-affinity antibodies is T cell—dependent, and T cells specific to GAD65, the neuronal antigen, are important in the T cell–B cell cognate interactions leading to B-cell differentiation to plasma cells secreting high-

affinity GAD65-ab. In this sense, GAD-specific T cells and clonally expanded GAD-specific B cells have been demonstrated to coexist intrathecally in patients with SPS, where they may collaborate in the synthesis of GAD-IgG.[116]

Other autoantibodies

Other possibility is that in these disorders, more relevant autoantibodies coexist with GAD-ab. A few other antigenic targets, particularly for SPS, have been identified; all localized in the pre- or post-synaptic compartment of inhibitory synapses. The figure 2 is a representation of almost all known autoantigens in the GABAergic synapsis found in patients with SPS. In paraneoplastic variants (5% of SPS patients), sera contained antibodies against amphiphysin (mainly breast or lung cancer) or against gephyrin in one patient with a mediastinum carcinoma.[117,118] Amphiphysin, as aforementioned, is a cytosolic pre-synaptic vesicle-associated protein with a role in endocytosis, whereas gephyrin is also cytosolic, but post-synaptic and it interacts with $GABA_A$ or glycine receptors and the cytoskeleton. So far, antibodies against gephyrin have not been found in other patients further than the index patient. In contrast, the presence of antibodies against amphiphysin is higher and their pathogenic role has been demonstrated. The disease was successfully transferred to rats using serum from a patient suffering from paraneoplastic SPS with high-titre antibodies against amphiphysin, as previously discussed.[99] Further experiments showed that anti-amphiphysin IgG was internalized into neurons co-localizing *in vivo* with pre-synaptic vesicular proteins, and reduced stimulated release of GABA through a structural disorganization in GABAergic synapses that ultimately lead to depletion of resting pool

vesicles and trapping of releasable pool vesicular proteins at the plasma membrane.[100,119] GAD-ab were not simultaneously found in these paraneoplastic cases.

Another target within the inhibitory synapse that has been associated to SPS, is the GABA receptor-associated protein (GABARAP). GABARAP is a microtubule-associated protein expressed in the cytosol and in axonal processes, and interacts with gephyrin to assemble the $GABA_A$ receptor ($GABA_AR$). Antibodies against GABARAP have been recently found by mass spectrometry in 70% of GAD65-positive SPS sera.[120] *In vitro* experiments demonstrated that the IgG from GABARAP antibody-positive patients, but not control IgG, significantly inhibited the surface expression of $GABA_AR$. Interestingly, 12 SPS patients in that study with severe disease had higher GABARAP-ab titres compared with eight patients with lower titres and a milder clinical severity, suggesting that GABARAP-ab may correlate with disease severity, unlike the GAD-ab. This finding has never been further replicated.

All the aforementioned antigens are intracellular. The group of antibodies against cell-surface antigens, a field developed basically in the last 10 years, is more interesting since many of them have been demonstrated to be pathogenic. Little evidence has been gathered in GAD-related neurological syndromes. In a cohort of GAD-ab-positive patients, when sera were adsorbed with purified GAD protein, binding activities against other neuronal surface antigens were demonstrated. The antigens involved in this reactivity remain yet unidentified.[106]

Our group identified in 2011 three patients with concomitant antibodies against the $GABA_B$-receptor among a cohort of patients with high titres of

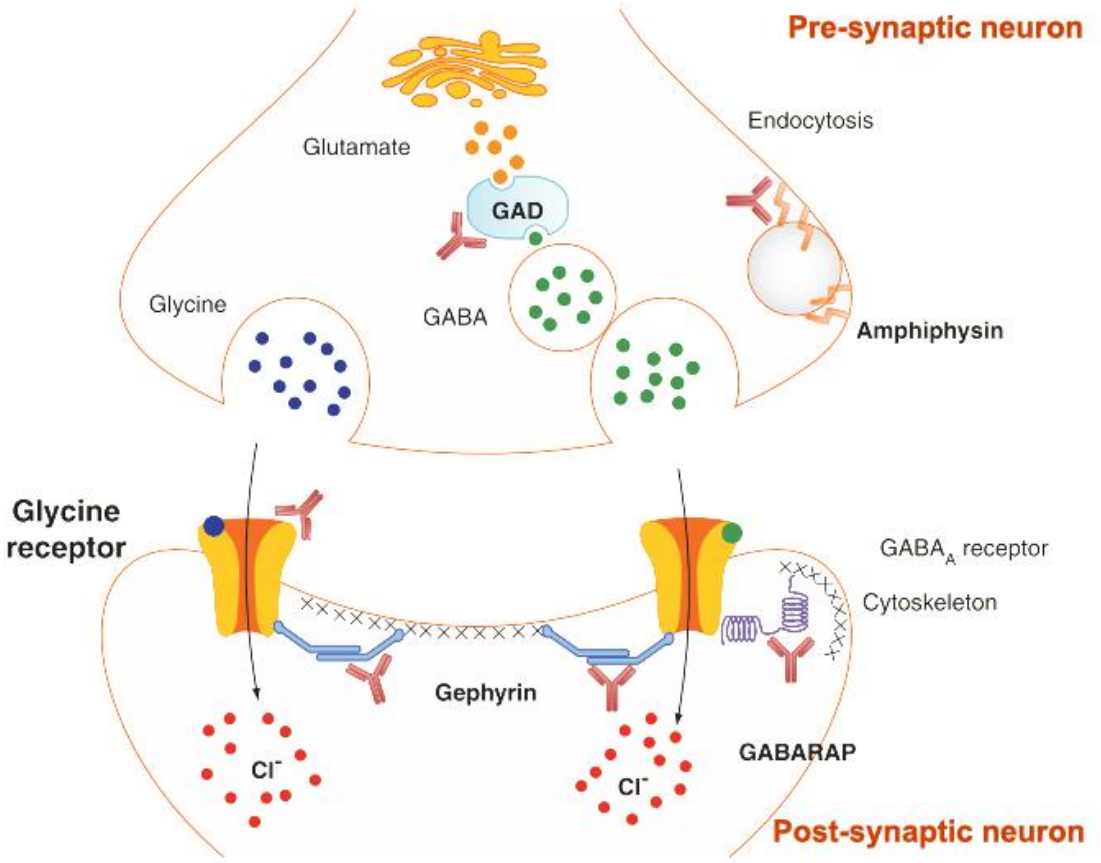
GAD-ab.[121] The interesting finding is that all $GABA_B$-receptor positive cases were paraneoplastic (two LE with lung cancer and one CA with thymus carcinoid).

Recently it has been identified antibodies directed against the alpha subunit of the glycine receptor (GlyRα1). Glycine is a key neurotransmitter in spinal inhibitory interneurons and glycine receptors are mostly expressed in the spinal cord, brainstem and cerebellum, and mutations in the glycine receptor can cause hereditary hyperekplexia in infants.[122] Antibodies against GlyR (GlyR-ab) were first described in PERM[123] and subsequently have been found in around 10% of patients with SPS.[124] Immunotherapy responses were noted more frequently in GlyR-ab–positive cases than in those patients without GlyR-ab. *In vitro*, the GlyR-ab activated complement on glycine receptor-transfected human embryonic kidney cells at room temperature, and caused internalization and lysosomal degradation of the glycine receptors.[125] It is unknown if they exist also in other GAD-related neurological disorders.

A third candidate antigen is the $GABA_AR$. In a recent work of our group, high serum and CSF titres of antibodies directed against the $GABA_AR$ were identified in patients who developed refractory status epilepticus or *epilepsia partialis continua* along with extensive cortical-subcortical MRI abnormalities, including 3 patients with GAD-ab. *In vitro*, patients' antibodies caused a selective reduction of $GABA_AR$ clusters at synapses.[126] The role of these antibodies in the pathogenesis of GAD-associated neurological condition is then unknown, but may be relevant in cases with epileptic symptoms.

This preliminary evidence suggests that additional neuronal autoantibodies may be present in the setting of GAD autoimmunity, at least in some subgroups of patients. However, a systematic investigation for neuronal cell surface autoantibodies has not been performed in GAD-positive patients, especially in those patients with other syndromes that are not SPS.

Fig.2. Autoantigens in the GABAergic synapsis in patients with GAD-associated neurological disorders



In the pre-synaptic neuron the main antigens are GAD, the enzyme converting glutamate to GABA and amphiphysin, a synaptic vesicle-associated protein responsible for endocytosis of vesicle plasma membranes following GABA release. In the post-synaptic neuron, the target antigens are glycine receptor, gephyrin and GABARAP. Glycine receptors are ligand-binding ion channels. Gephyrin is a cytoskeleton-binding protein needed for clustering post-synaptic receptors. GABARAP is a linker protein, involved in the organization of the $GABA_A$-receptors. $GABA_B$-receptor is not represented.

GABARAP: $GABA_A$-receptor-associated protein; GAD: Glutamic acid decarboxylase

Adapted from *Alexopoulos H, Dalakas MC. Immunology of stiff person syndrome and other GAD-associated neurological disorders. Expert Rev Clin Immunol. 2013 Nov;9(11):1043-53*.

Syndrome-specificity of neuronal autoantibodies

Immune responses that are more pathogenically relevant (autoantibody-mediated) generally show a high degree of syndrome specificity. The antibody, after binding its target, prompts a unique or restricted phenotype. In line with other concerns about the pathogenic role, GAD autoimmunity associates with a variety of neurological syndromes that may co-exist in the same patient in rare occasions. GAD-ab appeared initially as a syndrome-specific antibody, due to the selection in initial studies of relatively homogeneous cohorts of patients with SPS. This consideration changed as new evidence emerged, demonstrating at the same time that very similar phenotypes can associate with different immune responses.

Same antibody, different neurological phenotypes

An intriguing issue is how patients with the same autoimmune biomarker develop such a striking different phenomenology. Why a patient with GAD-ab develops a SPS, which implies a predominant involvement of the spinal cord and brainstem, whereas another patient presents with limbic encephalitis or resistant epilepsy due to mesotemporal lobe involvement.

The most comprehensive attempts to find phenotypic determinants in the immune response are the investigations comparing the serum from patients with T1DM and SPS. Purified IgG from patients with CNS disorders produce a selective suppression of GABAergic transmission *in vitro*. In contrast, IgG from diabetic or polyendocrine patients do not alter the GABAergic function unless in exceptional cases with high titres of GAD-ab.[81,82]. In addition to different titers, the immune response to GAD have

other differential features: a distinct epitope-specificity in the recognition of GAD65, and a different prevalence of antibodies against GAD67 in addition to GAD65-ab. Extended research showed that in both conditions the humoral response is complex since the antibodies recognize several regions of the GAD65, but there are some immunodominant epitopes. In SPS the antibodies are primarily against linear epitopes located predominantly in the middle domain of the protein, and less frequently in the C- or NH-terminal domains; whereas in T1DM are primarily directed against conformational epitopes almost exclusively located in the middle and C-terminal domains.[127–129] Also investigations in auto-reactive T cells have shown a different epitope specificity. In a series of eight patients, T cells from peripheral blood of SPS patients recognized two dominant epitopes from GAD65 (aa 81–171 and aa 313–404), which were different from those seen in T1DM.[111,112] The second differential feature is the prevalence of antibodies against both isoforms at the same time. Whereas in patients with T1DM the detection of concomitant GAD67-ab is around one third of the patients, in SPS the proportion of GAD67-ab in sera is up to 70%. Considering that GAD67 in not expressed in human pancreatic cells, their detection could be surprising. Further studies demonstrated that the presence of these antibodies represent a minor population of GAD65-ab that are reactive with a cross-reactive epitope found also on GAD67;[129–131] in neurological disorders their significance is not clear and it is unknown their prevalence among other GAD-associated neurological disorders.[128,132,133]

Recent publications suggest that analogous disparities in epitope specificities could be relevant in the neurological presentation. In a number of experiments was shown different epitope-recognition among GAD-ab

from different neurological syndromes[105] and a different functional effect of the antibodies depending on the syndrome. In particular, GAD-ab from a patient with cerebellar ataxia had different neurophysiological and neurochemical effects than GAD-ab from patients with SPS after intra-cerebellar administration in rats;[134] and studies *in vitro* using sera or CSF form patients with LE do not affect GABAergic neurotransmission in cultured hippocampal networks, as has been demonstrated in SPS or cerebellar ataxia.[135–137] The majority of these studies are based in the same epitope mapping approach, by means of competition binding assays using 2 monoclonal antibodies: b78 and b96.11. These monoclonal antibodies recognize different epitopes located at the C-terminus of GAD65, present different ability to inhibit the enzymatic activity *in vitro* (only b78 is able to do it) and different behavioral effects (b96.11 provoked immediate severe effects, which rapidly decrease, whereas b78 induce moderate but prolonged effects in procedural spatial functions based on the Morris Water Maze test performance[98]). Nevertheless, this epitope mapping is indirect, and alternative methods using constructs or deleted mutants spanning the whole protein, similar to the former studies with sera from T1DM or SPS, are lacking.

Moreover, this dissimilar functional effect (*in vitro* or in animal models) of serum or CSF IgG depending on the type of neurological syndrome could be due to the presence of additional autoantibodies (previously discussed), especially those against neuronal cell-surface, whose effects have been demonstrated *in vitro*.[125,126] *To date there are no large series or comprehensive studies comparing the spectrum and heterogeneity of the immune responses that occur in patients with diverse GAD-associated syndromes.* Furthermore, it is unclear the contribution of other additional

autoantibodies in the clinical course or co-morbidities. Besides a potential pathogenic role in GAD-associated neurological conditions, they may have clinical implications. In fact, additional antibodies against $GABA_B$-R in the context of GAD-associated neurological disorders have been found only in patients with a tumor[121]; and in patients with SPS and GAD-ab, the presence of additional antibodies against GlyR are associated with a better response to immunotherapy.[124]

Same neurological phenotype, different antibodies.

The stiff-person syndrome (classic phenotype and its variants) is a great example of how different autoimmune processes can associate a similar phenotype. Recent investigations have shown that it is a heterogeneous immunological condition. This constellation of phenotypes can be collected under the term stiff-person spectrum disorders (SPSD), in parallelism with other group of autoimmune disorders of the CNS that present overlapping symptoms but different underlying autoimmunities, such as the neuromyelitis optica spectrum disorders.

Besides marking an autoimmune aetiology, if the detection of particular antibodies has additional value over the clinical classification is unknown. Three independent series of patients with SPSD collected together a total number of 190 patients, that were classified in 3 groups based on clinical and electrophysiological criteria, and investigated their immunological status.[31,45,49] Fifty patients were classified as partial SPS (or *stiff-limb syndrome*) and 26 (52%) had GAD-ab. In contrast, among the 106 patients classified as classic SPS, 89 (84%) had GAD-ab.[49] Among those patients that were GAD-ab-negative, 5 had antibodies against amphiphysin. This finding

is almost invariably associated with a hidden breast cancer and patients are characterized by a particular presentation consisting in rapid progression, with upper limb involvement and the development of fixed deformity of a limb.[138] The third group of patients was classified as having progressive encephalomyelitis with rigidity and myoclonus (PERM); 23 out of 34 (68%) had GAD-ab. These patients are characterized by rigidity that typically involves upper or distal limbs with abnormal postures and generalized myoclonus, frequent brainstem dysfunction and prominent dysautonomia. Before the introduction of immunotherapy in the management of these patients, the outcome was invariably lethal before 2-3 years. In these large series, the response to immunomodulatory therapies was not dependent on antibody status (presence or absence of GAD-ab or antibodies against amphiphysin), antibody level, or the clinical classification.

In 2008, years after this large series exploring the clinic-immunological associations, Hutchison et al. described a patient with PERM, without GAD-ab, who presented a delayed but substantial recover after immunotherapy. The serum of this patient was found to contain antibodies recognizing the glycine receptor. A recent study in a cohort of patients GlyRα1-positive found that 73% had a phenotype of PERM, based on author's criteria.[125] Nevertheless, GlyR-ab have been found in 10-15% patients with SPS, sometimes in coexistence with GAD-ab.[124,139] The most interesting association of GlyR-ab detection in this studies is not to a specific clinical phenotype, but a good response to immunotherapy. In analogy to what have been described in other neurological disorders associated with antibodies against cell-surface antigens, a good outcome was observed in many severe cases of PERM with GlyR-ab after immunotherapy.[125]

SPSD is thus a complex autoimmune entity, and can occur with several autoantibodies. Since the antibodies are not a mandatory diagnostic criterion, determination of antineuronal antibodies are not part of the initial evaluation in many patients. Although *preliminary evidence suggests that autoantibodies may have prognostic implications in SPSD, a comprehensive study including different clinical phenotypes has not been performed yet*.

Hypothesis and objectives

Hypothesis

High serum levels of GAD-ab are biomarkers of several neurological syndromes. Stiff-person syndrome is the most frequent, followed by cerebellar ataxia, limbic encephalitis, epilepsy and other less frequent neurological syndromes. The role of GAD-ab is still a matter of debate. Experimental studies suggest that GAD-ab may have different effect *in vivo/in vitro* depending on a distinct epitope-recognition specificity within the antigen, but their pathogenicity has not been definitely proven. In the search for more relevant autoantibodies, different antibodies have been demonstrated in some patients with GAD-associated neurological disorders, including those against neuronal cell-surface or synaptic antigens. These additional antibodies had clinical implications in the cases described, like a great response to immunotherapy in patients with stiff-person spectrum disorders or association with cancer.

GAD-ab associate with different neurological syndromes, rarely present in the same patient. The heterogeneity of the immunologic response may be responsible for the diversity of neurological phenotypes and, additionally, may have prognostic implications in particular syndromes.

In the setting of GAD autoimmunity, there are certain conditions such as the presence of an underlying tumour, or the depletion of Purkinje cells in cerebellar ataxia, which could implicate a worse prognosis if the diagnosis is delayed. Predictive factors of cancer or treatment response are unclear.

Objectives

1. To investigate if there are any differences in the immunological profile in serum and CSF from patients with GAD-ab and different neurological syndromes (stiff-person syndrome, cerebellar ataxia, epilepsy or limbic encephalitis). In particular:

    a. To study the humoral response against the GAD isoforms

    b. To study the presence of co-existing antibodies to other proteins or receptors of the inhibitory synapses

2. To describe the long-term effect of the immunotherapy, and to investigate predictive factors of response in a cohort of patients with cerebellar ataxia and GAD-ab

3. To investigate the clinical and immunological features of patients with neurological syndromes, GAD-ab, and the presence of a tumour, and to explore the predictive factors associated with risk of cancer

4. To determine the value of GAD-ab in a cohort of patients with stiff-person spectrum disorders

Publications

Paper I

Antibodies to Inhibitory Synaptic Proteins in Neurological Syndromes Associated with Glutamic Acid Decarboxylase Autoimmunity.

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(* These authors contributed equally to this work)

IF JCR 2015 (percentile): 3.057 (Q1)

Contribution:

This is the most comprehensive study looking for immunologic determinants of the phenotypic diversity in GAD-associated neurological syndromes. Samples from 106 patients were included in the study

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RESEARCH ARTICLE

Antibodies to Inhibitory Synaptic Proteins in Neurological Syndromes Associated with Glutamic Acid Decarboxylase Autoimmunity

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Abstract

Antibodies to glutamic acid decarboxylase (GAD-ab) associate to different neurological syndromes. It is unknown if the diversity in syndrome association represents epitopes in different immunodominant domains or co-existence of antibodies to other proteins of the inhibitory synapsis. We examined the serum and CSF of 106 patients with anti-GAD related syndromes (39 cerebellar ataxia, 32 stiff-person syndrome [SPS], 18 epilepsy, and 17 limbic encephalitis [LE]). GAD65-ab titres were quantified by ELISA. Immunoblot was used to determine if the antibody-targeted epitopes of GAD65 and GAD67 were linear. A cell-based assay (CBA) with HEK293 cells expressing the GAD65 N-terminal, central catalytic domain, or C-terminal was used to investigate the immunodominant domains. Antibodies to GAD67, gamma-aminobutyric acid A receptor (GABAaR), glycine receptor (GlyR), GABAaR-associated protein (GABARAP), and gephyrin were determined with CBA. GAD-ab internalization was investigated using cultured rat hippocampal neurons. CSF GAD65-ab titres were higher in patients with cerebellar ataxia and LE compared to those with SPS ($p = 0.02$). GAD67-ab were identified in 81% of sera and 100% of CSF. GAD65-ab recognized linear epitopes in 98% of the patients and GAD67-ab in 42% ($p<0.001$). The GAD65 catalytic domain was recognized by 93% of sera, and the three domains by 22% of sera and 74% of CSF ($p<0.001$). Six patients had GABAaR-ab and another 6 had GlyR-ab without association to distinctive symptoms. None of the patients had gephyrin- or GABARAP-ab. GAD65-ab were not internalized by live neurons. Overall, these findings show that regardless of the neurological syndrome, the CSF immune response against GAD is more widespread than that of the serum and that there is no specific association between clinical phenotype and the presence of antibodies against other proteins of the inhibitory synapsis.

Competing Interests: Dr. Dalmau has a research grant from Euroimmun, and receives royalties from patents for the use of the patent concerning anti-Ma antibodies (Patent Number: US 6,387,639; Issued May 14th, 2002. Licensed to Athena Diagnostics, USA) and the one for NMDAR antibodies (Patent Name: NMDAR test; USA Patent Number: US 7,972,796; Issued July 5th, 2011. Licensed to Euroimmun, Inc, Germany. Europe patent number: EP 2 057 466). The other authors report no disclosures. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

Introduction

High levels of antibodies against glutamic acid decarboxylase (GAD-ab) have been reported in serum of patients with several neurological syndromes, including stiff person syndrome (SPS), cerebellar ataxia, epilepsy, and limbic encephalitis (LE), all of them characterized by neurological dysfunction of the GABAergic system [1–3]. The reason why some patients develop one neurological syndrome versus another is unclear. Neurological syndromes linked to GAD-ab were initially described in 1988 [4] but to date there are no large series or comprehensive studies comparing the spectrum and heterogeneity of the immune responses that occur in patients with diverse anti-GAD-associated syndromes. Studies addressing this issue are small or restricted to SPS, predominantly focused on the GAD65 isoform, or using only serum. In addition, it was postulated that in patients with GAD-ab and LE or seizures, these symptoms could be caused by more relevant autoantibodies against cell surface antigens and respond well to immunotherapy [5]. On the other hand, there are patients with LE and isolated GAD-ab that appear to have worse outcome [6]. Therefore, determination of whether patients with different anti-GAD associated syndromes have distinct underlying immune responses may have practical clinical implications.

The pathogenic significance of GAD65-ab is controversial. Some studies suggest they play a direct pathogenic role, but several lines of evidence suggest otherwise. First, GAD65-ab-positive neurological syndromes do not respond well to immunotherapy compared to those associated with antibodies against neuronal surface antigens [7,8], second, there is no correlation between antibody titres and disease severity [9], and third, there are no convincing animal models of the neurological disorders [10,11]. An important step towards proof of pathogenicity would be the demonstration that GAD-ab bind to live neurons, and after internalization reach the intracellular GAD isoforms.

To address all these questions, we examined serum or CSF of 106 patients with different anti-GAD associated neurological syndromes aiming to determine the repertoires of antibodies against the two GAD isoforms, the main immunodominant regions and linear or conformational structure of the epitopes, the presence of co-existing antibodies to other proteins or receptors of the inhibitory synapses, and whether GAD-ab were internalized by live neurons.

Materials and Methods

Patients and inclusion criteria

Patients were seen by the authors or referring physicians between December 1994 and April 2013. Serum or CSF were examined for autoantibodies in the laboratory of Neuroimmunology at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clinic, Barcelona, Spain, or in the Department of Neurology, Hospital of the University of Pennsylvania, Philadelphia, USA. Inclusion criteria was the presentation of a neurological disorder associated with serum GAD65-ab detected by brain immunohistochemistry (this technique detects GAD-ab with radioimmunoassay (RIA) levels >2000U/mL; patients below these titres have diabetes (T1DM), but almost never neurological symptoms) [1] and confirmed by cell-based assay (CBA) of HEK293 cells expressing GAD65. Patients with a definite or possible paraneoplastic syndrome were excluded from the study [12].

Clinical information was obtained directly by the authors or provided by referring physicians, through questionnaires and telephone interviews. SPS included patients with classical forms, defined by stiffness and spasms predominantly involving the proximal aspect of the legs and lumbar muscles, and patients with partial forms, including stiff-limb syndrome or isolated lower or upper extremity stiffness [13]. Cerebellar ataxia was considered in patients who

Table 1. Basic clinical information of the cohort study.

	Cerebellar ataxia	Stiff-person syndrome	Isolated epilepsy	Limbic encephalitis
Patients, (CSF)	39 (26)	32 (17)	18 (8)	17 (10)
Female, (%)	32 (82)	29 (91)	15/17 (88)	12/15 (80)
Age (in years), median (range)	60 (32–79)	53 (5–77)	32 (9–67)	26 (12–49)
Overlapping syndrome	14 (9[a]+5[b])	5 (3[b]+2[c])	0	1[a]
T1DM at onset, (%)	12/32 (38)	14/29 (48)	4/9 (44)	2/6 (33)
Thyroiditis, (%)	18/30 (60)	7/25 (28)	7/10 (70)	3/5 (60)
Other autoimmune disorders[d]	9	5	3	2
CSF oligoclonal bands, (%)	18/24 (75)	4/15 (27)	4/9 (44)	7/7 (100)
Months from onset to GAD diagnosis, median (IQR)	4 (1–11)	36 (16–72)	19 (1–72)	12 (7–15)
Immunotherapy				
Steroids (+ IVIg)	4 (3)	2 (2)	0	5 (2)
Other combinations[e]	3	4	0	0

IQR: interquartile range; IVIg: intravenous immunoglobulin; T1DM: type 1 diabetes mellitus.

[a] Coexistent stiff-person syndrome

[b] Epilepsy (5 of them drug-resistant temporal lobe epilepsy)

[c] Cerebellar ataxia

[d] Pernicious anemia: 10 patients, celiac disease: 2, vitiligo: 5, psoriasis: 2, myasthenia: 2

[e] IVIg or plasma exchange: 4; azathioprine: 2; IVIg + rituximab: 1.

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developed a cerebellar syndrome and fulfilled previously reported criteria (absence of alternative explanation and high GAD65-ab levels) [8]. LE was defined by the subacute onset of short-term memory loss, behaviour change, seizures, and involvement of the temporal lobes by imaging studies, or post-mortem examination [12,14]. Patients who developed isolated epilepsy without neuroimaging criteria of limbic involvement were classified as epileptic patients. When several GAD-associated neurological syndromes coexisted in a patient, the predominant syndrome by the time of diagnosis was used to classify such patient. Overall, 106 patients where included in the study. Four groups with different neurological syndromes were identified: 39 with cerebellar ataxia (CSF samples: 26), 32 with SPS (17), 17 with LE cases (10), and 18 with epilepsy (8). Table 1 summarizes the clinical characteristics of the study cohort.

Standard Protocol Approvals and Patient Consents

All subjects gave written informed consent for the storage and use of serum and CSF samples for research purposes. Serum and CSF samples used in the study are deposited in the collection of biological samples named "Neuroinmunología" registered in the biobank of Institut d' Investigació Biomèdica August Pi i Sunyer (IDIBAPS), Barcelona, Spain. Animal handling procedures were approved by the Local Ethics Committee (99/1 University of Barcelona) and the Generalitat de Catalunya (1094/99), in accordance with the Directive 86/609/ EU of the European Commission. The study was approved by the Comité Ètic d'Investigació Clínica (CEIC) of Hospital Clinic.

Immunohistochemistry of rat cerebellum

GAD-ab immunoreactivity was analyzed (serum screening dilution 1:500; CSF undiluted) using an avidin-biotin technique on paraformaldehyde-fixed frozen rat cerebellar sections as described previously [15]. To study the distribution of IgG subclasses of the antibody, the same

immunohistochemistry technique was used after changing the secondary antibody by biotinylated mouse monoclonal antibodies to human IgG 1–4 subclasses (Sigma, St. Louis, MO) (dilutions: anti-IgG1 1:100, anti IgG2 1:200, anti-IgG3 1:200, and anti-IgG4 1:200) as described [16].

To study the presence of intrathecal synthesis of GAD-ab, the GAD-ab titers obtained by immunohistochemistry in paired samples of CSF and serum and the CSF/serum albumin index were used to calculate the index for intrathecal synthesis of GAD-ab as previously reported [1]. Values higher than 1, are a strong indicator of intrathecal synthesis of antibody-specific IgG [17].

GAD65 ELISA

Levels of GAD65-ab were detected by ELISA (RSR Limited, UK) using a commercial kit following manufacturer's specifications. Since GAD65 titres in neurologic syndromes are high, serums and CSF were titrated to determine the optimal dilution factor. Briefly, ELISA wells were seeded for 1 h with patients' sera diluted 1:10000 or CSF diluted 1:200, followed by 1h incubation with GAD65 biotinylated protein, and 20 min incubation with streptavidin peroxidase. Colorimetric reaction was developed with 3,3',5,5'-Tetramethylbenzidine for 20min, stopped with sulfuric acid and read at 450nm in a multiplate reader.

Immunoblot

Human GAD65 (Diamyd, Stockholm, Sweden), GAD67 (Abnova, Taipei, Taiwan), and GABARAP (Abnova) recombinant proteins were electrophoretically separated and transferred to a polyvinylidene difluoride membrane and strips incubated with the patient's serum (1:1000 dilution), GAD65 (GAD-6, Hybridoma Bank, Iowa City, IA), GAD67 (Abcam, Cambridge, UK), or GABARAP (Abcam) commercial antibodies followed by biotinylated goat antihuman IgG or horse antimouse IgG (Vector Laboratories, Burlingame, CA) and developed with diaminobenzidine tetrahyrochloride [15]. In some experiments we used an enhanced chimioluminiscence technique (ECL Western Blotting System) following manufacturer's instructions. The specificity of GAD65 and GAD67 commercial antibodies was confirmed with immunoblots with recombinant proteins and also with HEK293 cells (see below) expressing the corresponding antigens.

Immunocytochemistry on HEK293 cells

To test the presence of antibodies against different synaptic antigens, HEK293 cells were transfected with plasmids containing human GAD65 or GAD67 (OriGene, Rockville, MD), the α1 and β3 subunits (co-transfected 1:1) of the GABAaR, GABARAP, α1 subunit of the GlyR, or gephyrin (co-transfected 1:1 with collybistin) as described [8,18–20]. All DNA sequences were purchased from OriGene except from those of GlyR, gephyrin and collybistin (a gift of Dr. RJ Harvey). Twenty-four hours after transfection, immunofluorescence on live (for the GABAaR and GlyR assays) or fixed (for the other assays) HEK293 cells was performed as described [5]. Briefly, HEK293 cells were incubated with patients' serum (1:40) or CSF (1:5) combined with the corresponding primary commercial antibodies, GAD65 (GAD-6, Hybridoma Bank), GAD67 (Abcam), anti-GABAa receptor α1 subunit (Millipore Termecula, CA), anti-glycine receptor α1 subunit (Synaptic systems, Göttingen, Germany), or GABARAP (Abcam), followed by incubation with goat anti-human IgG Alexa Fluor 488 or goat anti-mouse IgG Alexa Fluor 594 (Life Technologies, Eugene, OR). When live HEK293 cells were used, they were fixed with 4% paraformaldehyde, and permeabilized with 0,3% triton X-100, after the incubation with patients' and commercial primary antibodies. Results were photographed under

fluorescence microscope (Zeiss Axioimager M2) using Zeiss Axiovision software (Zeiss, Oberkochen, Germany).

To validate the CBA for GAD65, 50 sera from patients with different neurological disorders (20 neurodegenerative, 15 NMDAR-ab, 10 anti-Hu, 5 other neuroimmunological disorders) and 17 from T1DM were used. None of the 50 sera were positive by this CBA and the sensitivity for T1DM depended on the titer: only 10/17 T1DM sera with higher antibody titer (1822–7900 U/ml by RIA) showed reactivity against GAD65. This finding indicates that the CBA for GAD65, like rat immunohistochemistry, is not sensitive enough to detect low levels of GAD65-ab (within the range typical of T1DM) and that are identified by RIA.

GAD65 epitope analysis

We designed plasmids to express the sequence of the N-terminal domain (Nt: aa 1–188), the central region containing the decarboxylase catalytic domain also termed pyridoxal-5'-phosphate (PLP) domain to indicate the cofactor used by GAD (aa 189–464), and the C-terminal domain (Ct: aa 465–585) from GAD65 (SC300136, OriGene). Customized sequences were ordered and commercially subcloned with NheI-NotI (GenScript, Hong Kong) into a pCMV6-AC-Myc-tagged plasmid (PS100003, OriGene) Resulting plasmids were transfected into HEK293 cells and immunocytochemistry studies were performed as described above. A commercial antibody against the Myc-Tag protein (Myc-Tag (9B11) mouse mAb, Cell Signaling Technology) was used to confirm the correct expression of each construct.

Analysis of antibodies against neuronal surface antigens

Antibodies against neuronal surface antigens were detected by immunohistochemistry of rat brain and immunofluorescence of rat hippocampal and cerebellar neuronal cultures as previously reported [5,21,22].

Study of internalization of GAD IgG

To determine if GAD-ab are internalized in vivo, GAD-ab-positive CSF samples diluted 1:25 were incubated with live rat hippocampal neurons for 24h at 37°C. Cells were then washed with fresh neurobasal media and incubated with unlabeled anti-human IgG antibody (Jackson Immunoresearch, West Grove, PA) at 1:5 dilution for 1h at 37°C (to block detection of any specific or non-specific human IgG binding to the neuronal cell surface). After extensive washing, neurons were fixed for 5min with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and incubated for 1h with Alexa Fluor 488 anti-human IgG. As positive control of the IgG internalization we used a NMDAR-ab-positive CSF. Results were photographed under confocal microscope (Zeiss LSM710) and analyzed with Zen software (Zen 2012 black edition 8.0, Zeiss).

Statistical analysis

Non-parametric tests (Fisher exact, W Wilcoxon) were used when the application conditions for parametric tests (χ^2, ANOVA) were violated. As ELISA values did not follow a normal distribution, log-transformation was used to normalize them prior to statistical analysis. Statistical software (Stata version 13; StataCorp) was used for the analyses and a p value < 0.05 was considered significant.

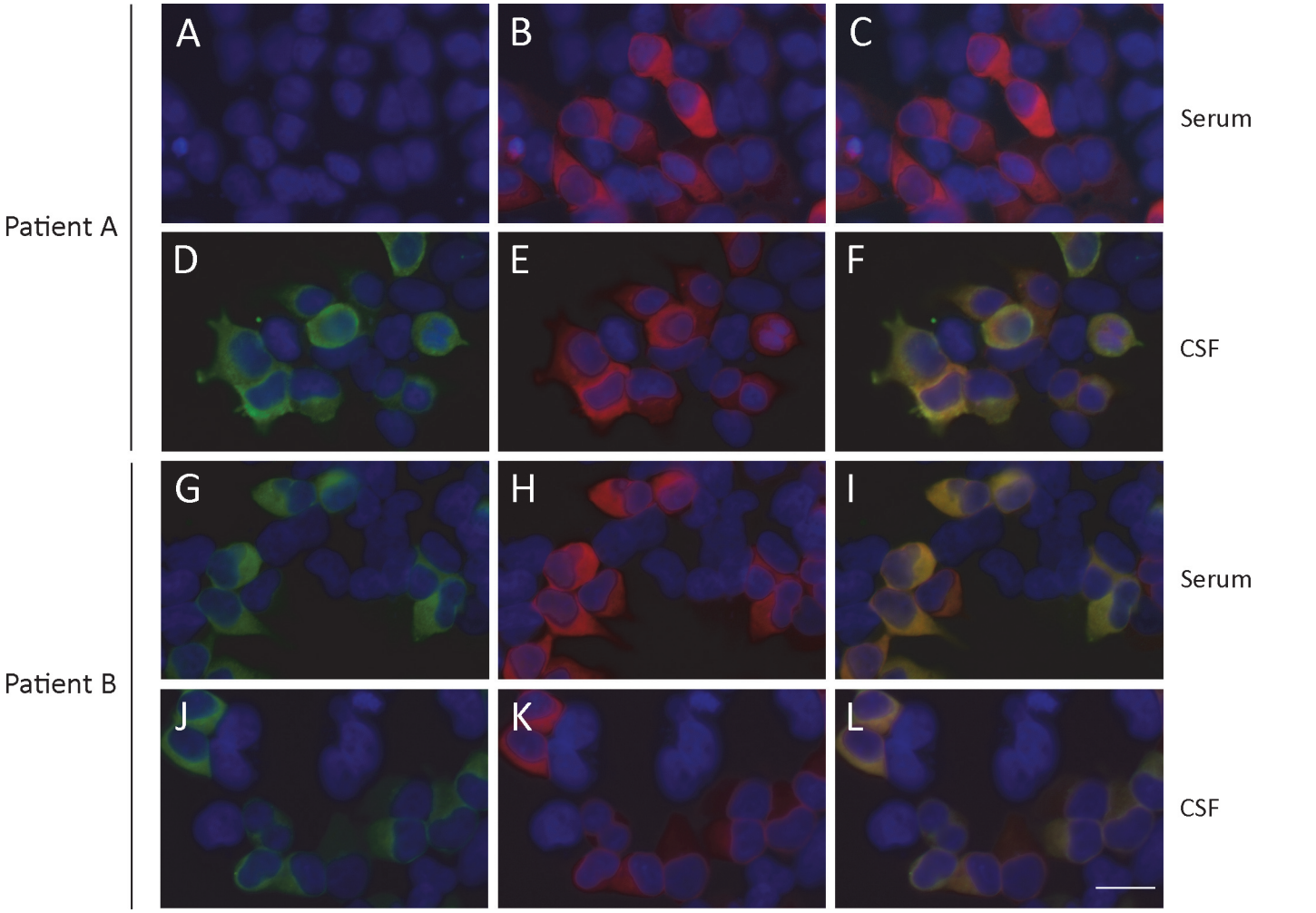

Fig 1. Reactivity of serum and CSF samples with HEK293 cells expressing GAD67. Fixed HEK293 cells transfected to express GAD67 were incubated with serum and CSF of patient A and B (in green) and a commercial antibody against GAD67 (in red). The nuclei of the cells are stained with 4′,6-diamidino-2-phenylindole (DAPI). The merged reactivity is shown in panels C, F, I, and L. The CSF, but not the serum, of patient A immunoreacted against GAD67, whereas the serum and CSF of patient B were both positive. Scale bar = 20μm.

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Results

By CBA, serum from the 106 (100%) patients had GAD65-ab and 88% GAD67-ab. No differences were observed in the frequency of GAD67-ab among the cerebellar ataxia, SPS, LE, or epilepsy groups. By contrast all 61 CSF tested (100%) contained both GAD65- and GAD67-ab (Fig. 1). There were nine paired serum-CSF samples in which the serum was negative and the CSF positive for GAD67-ab. These samples were equally distributed among the four clinical groups. Immunohistochemical analysis of IgG subclasses showed that in all 106 patients the predominant GAD immunoreactivity was IgG1.

Serum GAD65-ab levels tended to be higher in patients with cerebellar ataxia and LE (Fig. 2A). This trend was confirmed in the CSF where GAD65-ab levels were significantly higher in patients with cerebellar ataxia and LE compared to those of SPS patients (Fig. 2B). In addition, the median index of intrathecal synthesis of GAD65-ab in available paired serum/CSF was higher in 14 patients with cerebellar ataxia (median: 9.7 [IQR: 3.6–16.6]) compared to that of 11 with SPS (5.3 [2.3–17.4]); $p = 0.38$. The presence of serum GAD67-ab was associated

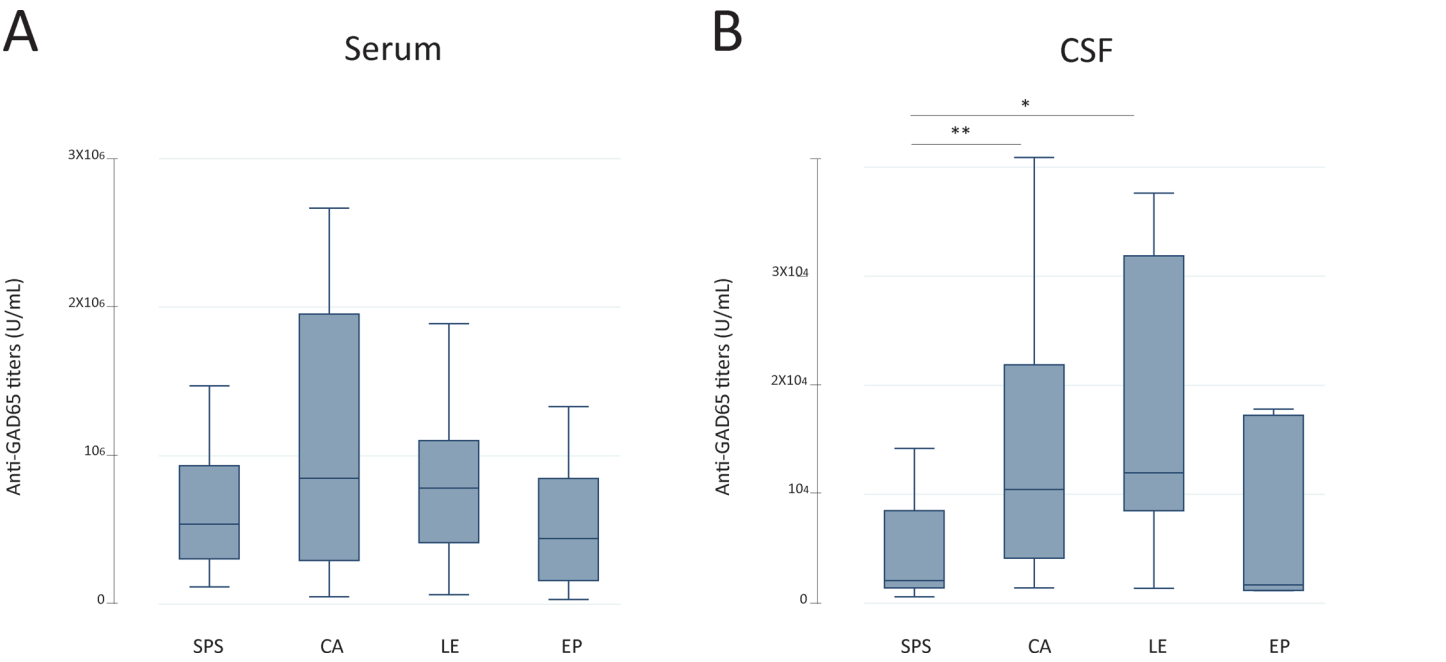

Fig 2. GAD65 antibody levels measured by ELISA in serum (A) and CSF (B) samples classified by neurological syndrome. CSF, but not serum, GAD65 antibody titres were significantly higher in the groups of ataxia and limbic encephalitis compared to those of stiff-person syndrome (median: 2.1 x10^3 U/ml (interquartile range (IQR):1.4–8.5) in SPS vs. 10.4 x10^3 U/ml (4.1–21.9) in CA; $^{**}p = 0.01$ and 12 x10^3 U/ml (8.5–31.9) in LE; $^{*}p = 0.02$). SPS: stiff person syndrome; CA: cerebellar ataxia; LE: limbic encephalitis; EP: epilepsy.

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with GAD65-ab titres (7.2 x10^5 U/ml (3.3–14.0) vs. 2.9 x10^5 U/ml (1.5–7.8) for patients with and without serum GAD67-ab respectively; $p = 0.02$; Fig. 3).

Analysis of GAD-ab epitopes

To elucidate whether GAD65- and GAD67-ab identify linear epitopes, all 106 serum samples were screened by immunoblot of purified GAD65 and GAD67 recombinant protein. All but two sera (98%) reacted with GAD65 whereas only 39 (42%) of the 93 sera with GAD67-ab assessed by the CBA were positive by immunoblot of GAD67 (Fig. 4). No correlation was found between the serum reactivity with GAD67 immunoblot and any specific neurological syndromes.

To investigate the antigenic region of GAD65 recognized by GAD65-ab of patients with the different neurological syndromes, we produced constructs of the three different domains of GAD65: Nt, PLP, and Ct. After demonstrating that the subcloning process did not induce a decrease in GAD65 immunoreactivity (data not shown), we then tested the reactivity of all sera and CSF samples on HEK293 cells expressing each of the three constructs. In 46% of patients serum GAD65-ab recognized only one domain, whereas 74% of CSF GAD65-ab recognized all three domains ($p < 0.001$) (Fig. 5). The most frequently recognized immunodominant region in serum was in the PLP domain; 93% of the sera showed reactivity against this region, including those with the lower titre of GAD65-ab. No correlation was noted between GAD65-ab levels and reactivity with any particular epitope. Serum of patients with LE were more likely to react with the Nt domain (69% vs. 29% for the rest of the patients; $p = 0.002$), whereas epileptic patients showed more reactivity against the Ct domain (67% vs. 38% for the other 3 groups; $p = 0.04$). We did not find these differences in CSF, since most of the samples recognized all three domains. No other epitope-region differences were seen among clinical phenotypes. The

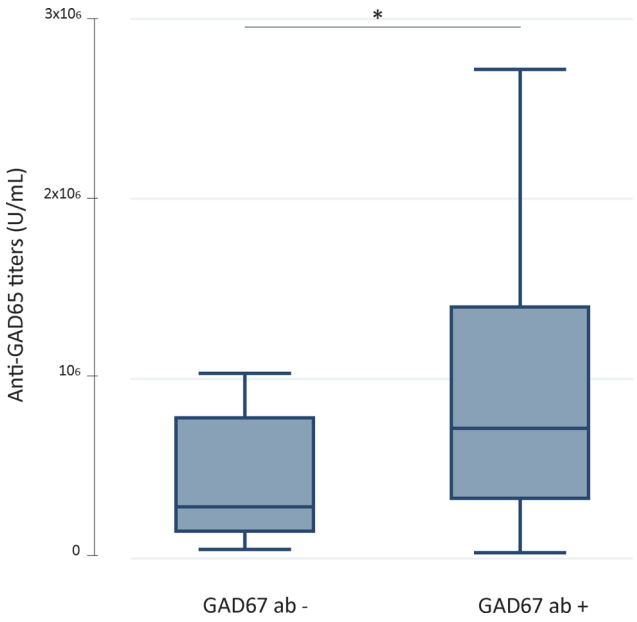

Fig 3. GAD65 antibody titres measured by ELISA in serum samples from patients with (n = 93) or without (n = 13) additional GAD67 antibodies. GAD65 antibody levels were higher in the group of patients with concurrent GAD67 antibodies. *p≤0.05.

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distribution of frequencies of the number of domains recognized by serum GAD65-ab was similar between the group of sera with or without GAD67-ab, indicating that the presence of GAD67-ab was not related to the extent of the reactivity against GAD65.

Presence of additional antibodies against the GABAergic inhibitory synapsis

All 106 serum and 61 CSF samples were examined by immunofluorescence with live rat hippocampal neurons (sera from cerebellar ataxia and stiff-person syndrome cases where also tested in cultures of cerebellar neurons), HEK293 cells expressing α1/β3 subunits of GABAaR, α1 subunit of GlyR, gephyrin or GABARAP. We found six patients with GABAaR antibodies (1 cerebellar ataxia, 2 SPS and 3 epilepsy, all in serum, CSF not available) and six with GlyR

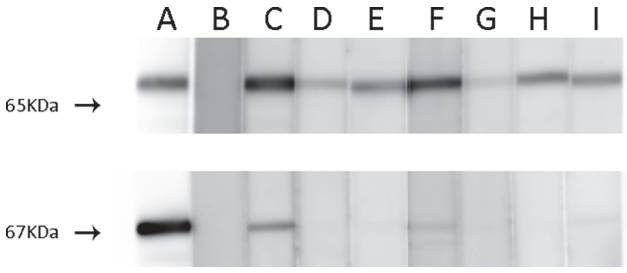

Fig 4. Immunoblot of purified GAD65 (upper panel) or GAD67 protein (lower panel). Strips were incubated with a commercial antibody (A), serum from a healthy individual (B), and serum from patients with cerebellar ataxia (C and D), stiff-person syndrome (E), limbic encephalitis (F and G) and epilepsy (H and I). All patients' sera reacted against GAD65 but only a few recognized GAD67 despite that all immunoreacted with HEK293 cells transfected with GAD67; this finding suggests that the recognized epitope is conformational. Both gels were run at the same time and blots were developed in parallel. The images were cropped to include the visible bands.

doi:10.1371/journal.pone.0121364.g004

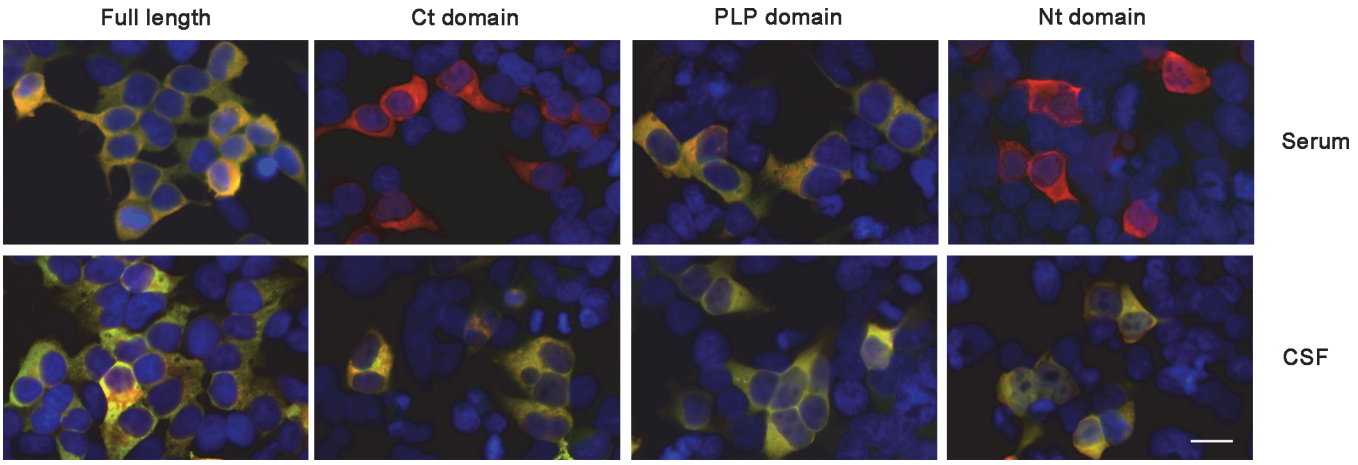

Fig 5. Study of GAD65 epitope immunoreactivity. Fixed HEK293 cells transfected to express full length GAD65, C-terminal (Ct), middle (PLP) and N-terminal (Nt) domains (all expressing an N-terminal myc tag) were incubated with patient serum (upper panels) or CSF (lower panels) (green) and a commercial antibody against myc tag (red). The nuclei of the cells are stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). The merged reactivity is shown in yellow. The serum sample reacts against full length and PLP domain, whereas the CSF sample shows a broader reactivity, staining the full length and three GAD65 domains. Scale bar = 20μm.

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antibodies (4 cerebellar and 2 epilepsy, all in serum, negative in the four CSF available). All samples had low titres (<1:200) of the antibodies. No additional antibodies against neuronal surface antigens were detected in neuronal cultures. We did not find any patient with GABARAP or gephyrin-ab. This result was confirmed by immunoblot using GABARAP recombinant proteins that were tested with serum from 32 patients with SPS (not shown).

Internalization of GAD-ab

If we postulate that GAD65 antibodies are pathogenic, they should reach the antigen, which is intracellular, and for this the antibodies need to be internalized. For this experiment we first confirmed that the cultured neurons used contained GAD65. Therefore, cultured neurons were first fixed, permeabilized and incubated with a commercial GAD-6 monoclonal antibody along with patients' CSF. This preliminary study indicated that the neurons contained intracytoplasmic GAD65 and that both, the commercial antibodies and patients' CSF antibodies were able to react with the antigen when the cell membrane was permeabilized (Fig. 6, panel A). Subsequently, we examined if patients' antibodies were able to react with live neurons (membrane not permeabilized). For this experiment live rat hippocampal neurons were incubated for 24 hours with GAD65 antibodies contained in the CSF of four patients with cerebellar ataxia, four with SPS, three with LE and five with epilepsy; the same experiment using CSF with NMDAR antibodies from a patient with anti-NMDA receptor encephalitis served as control of IgG internalization. No intracellular staining was detected in any of the four groups of patients with GAD-ab whereas IgG internalization was observed with the CSF of the patient with NMDAR-ab as previously reported [23] (Fig. 6). Overall, these set of studies demonstrate that patients ´ GAD65 antibodies only react with GAD65 when the membrane is permeabilized but not in live neurons.

Discussion

The current study provides for first time a comprehensive overview of the humoral immune responses present in serum and CSF of patients with neurological disorders associated with GAD-ab. Our data show several important immunological features; 1) the humoral immune

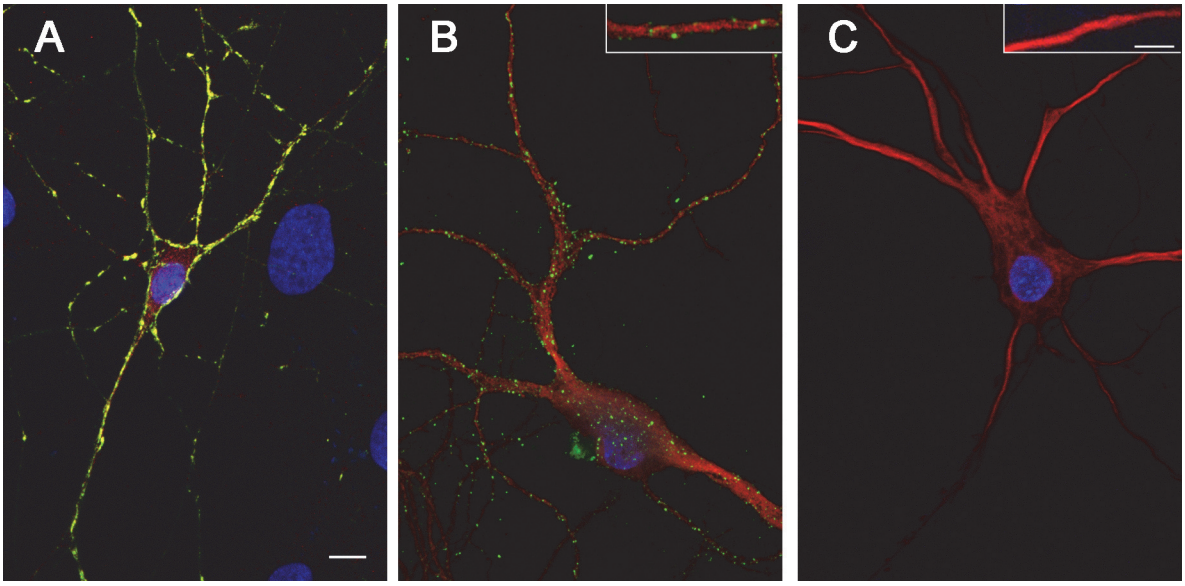

Fig 6. Study of anti-GAD IgG internalization in cultures of dissociated rat hippocampal neurons that express GAD65. Panel A demonstrates that neurons express GAD65; in this experiment neurons were permeabilized and incubated with a patient's CSF with GAD65-ab (green) and a commercial GAD65-ab (red); the co-localization of reactivity with GAD65 is shown in yellow. Panels B and C show the experiment of internalization; in B, live neurons were incubated with CSF of a patient with anti-NMDAR antibodies, and in C with the CSF of a patient with GAD65 antibodies for 24h at room temperature. After blocking the extracellular IgG binding with a secondary antibody without fluorescent tag, the neurons were washed, fixed and permeabilized and the internalized IgG was determined with a secondary anti-human IgG antibody with a fluorescence tag (green), or with an antibody against MAP2 (red). Insets show dendrites at higher magnification. Only the CSF of the patient with IgG antibodies against NMDAR showed IgG internalization, as previously reported (Hughes *et al*. 2010), and used here as a control. Scale bar = 10μm, insert scale bar = 2,5μm.

doi:10.1371/journal.pone.0121364.g006

response against GAD is different in CSF and serum. In the CSF the antibodies target more domains of GAD65 and also immunoreact with GAD67, 2) GAD65-ab titres were higher in the CSF of patients with LE or cerebellar ataxia compared to those with SPS, 3) the frequency of additional antibodies against antigens of the GABAergic inhibitory synapses is low across all neurological syndromes, and 4) GAD-ab are not internalized by primary neuronal cultures suggesting that GAD-ab are not directly pathogenic.

A novel finding of our study is that the humoral response to GAD was different in CSF and serum. All patients regardless of the associated neurological syndrome had GAD67-ab in the CSF, including 9/61 (15%) patients whose serum was negative for these antibodies. In addition, CSF GAD65-ab usually recognized epitopes present in the three domains examined. These data along with the frequent detection of CSF oligoclonal bands [24,25] and specific intrathecal synthesis of GAD65-ab [1,17,26] strongly suggest that the presence of CSF GAD65-ab is not only a result of passive antibody transfer from serum to CSF, but they are part of an active immune response orchestrated in the CNS compartment.

Although we did not identify differences in the GAD65 epitope repertoire among the four neurological syndromes examined, CSF levels of GAD65-ab were lower in patients with SPS than in those with cerebellar ataxia or LE which unlike SPS, commonly associate with neuronal loss[6,27]. In a previous series of five patients with cerebellar ataxia, the CSF GAD65-ab titres were similar to those of 10 patients with SPS. However, patients with cerebellar ataxia showed a 2.5-fold higher intrathecal production of GAD65-ab compared to SPS[28] as we have also found in the current study.

Analysis of GAD65-ab epitopes has been previously done in patients with T1DM and SPS but not in other neurological syndromes. In T1DM, GAD65-ab mostly recognize

conformational epitopes in the PLP and Ct domains [29,30]. Initial epitope studies in cohorts of SPS found that the strongest reactivity with GAD65 depended on two discontinuous domains in the Ct, and in addition most sera recognized at least one epitope in the Nt domain [31]. Other authors also described a dominant linear epitope located in the Nt domain [32,33]. In the present study, the PLP domain was the GAD65 region most frequently recognized by serum GAD65-ab with no differences among neurological syndromes. Our findings are similar to those of a recent study that using a luciferase immunoprecipitation technique and GAD65 sub-fragments deletion mutants showed that the central region containing the decarboxylase catalytic domain was highly immunoreactive with sera of all patients with SPS [34]. We did not find a significant difference in GAD65 immunodominant regions in patients with the four indicated neurological syndromes. Our analysis of immunodominant domains included three large GAD65 deletion constructs spanning the entire molecule. This analysis could have missed smaller protein segments containing epitopes specific for a particular neurological syndrome [35].

We did not find antibodies against gephyrin and GABARAP. Gephyrin is a protein involved in clustering both GlyR and GABAaR [36] and antibodies to this protein were described in a single patient with SPS and mediastinal cancer [37]. Antibodies to GABARAP, a protein that stabilizes GABAaR in the membrane [38], were described in 70% of a cohort of 27 patients with SPS and GAD65-ab [39]. It is unclear why we did not detect GABARAP-ab but the study published in 2006 has never been replicated. The methods of detection were different; while GABARAP-ab were initially detected by immunoprecipitation and immunoblot, we have used CBA for all our patients and immunoblot with recombinant GABARAP in a subgroup randomly selected. We cannot rule out that our techniques were less sensitive but if this was the case the levels of GABARAP antibodies should be very low.

The recent discovery of antibodies against cell-surface or synaptic receptors in patients with autoimmune encephalitis, along with data supporting their pathogenicity and sometimes coexistence with GAD-ab, led us to investigate whether additional cell-surface antibodies could offer an explanation for the syndrome diversity. In the current study we used the same approach that resulted in the discovery of 9 of 11 cell surface or synaptic receptor autoantigens [40] and only identified 12 cases (11%) with these antibodies, all directed against known target antigens (six GABAaR and six GlyR) and with a similar distribution among clinical phenotypes. These findings are therefore different from those of a recent study [41] that identified antibodies against unknown cell surface antigens in patients with GAD65-ab. We did not find coexisting GlyR-ab in patients with SPS; this is in contrast with a previous report that showed that 15% of patients had coexisting GAD65 and GlyR antibodies [42]. If different serum dilutions or other technical details are responsible for this difference is unclear; we used a similar live cell-based assay but the serum dilution was not provided in the previous study[42].

Lastly, the pathogenic role of GAD-ab has been subjected to debate [3]. In vitro studies have demonstrated that GAD-ab from SPS patients, but not from diabetic patients, inhibit the enzymatic activity of GAD65 [43]. Intrathecal and intracerebellar injections of IgG purified from GAD65-ab-positive samples of patients with SPS or cerebellar ataxia, or human monoclonal antibodies to GAD65, induced clinical symptoms and neurophysiological changes similar to those seen in patients [10,11,35,44]. However, these experiments failed to unambiguously demonstrate that the observed changes were directly related to the interaction of the antibodies with GAD65. Similarly, active immunization experiments with GAD65 did not cause neurological dysfunction in immunized animals [45,46]. GAD65 is an intracellular antigen and therefore an essential experiment is the demonstration that GAD-ab are internalized and reach the antigen. Previous studies showed that human monoclonal antibodies to GAD65 were internalized by cells from an immortalized rat CNS cell line [44] or into neurons surrounding the

area of injection into rat cerebral cortex[47]. However, none of these studies showed that the antibodies reacted with intracellular GAD in vivo [44,47]. Our in vitro experiments failed to show that GAD-ab are internalized in cultures of neurons that express GAD65 casting doubt on the antibody pathogenicity. These experiments are nor definite proof that GAD-ab are not pathogenic; however, to resolve this issue future studies should unambiguously demonstrate that GAD-ab can reach the target antigen, visualizing the binding with high resolution confocal microscopy, and determining if the potential effects on GAD correlate with symptoms using appropriate animal models, as recently demonstrated for NMDAR antibodies in patients with anti-NMDAR encephalitis [48].

Conclusions

The present study emphasizes the importance of studying the CSF of patients with CNS syndromes suspected to be mediated by GAD autoimmunity because the repertoire of antibodies to different immunodominant regions is wider in the CNS than systemically. We did not find a specific immunodominant region or associated antibody that could be used as a biomarker of a particular neurological anti-GAD-associated syndrome. Moreover, GAD-ab are not internalized in live neurons, raising doubts about the direct pathogenicity of the antibodies.

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Author Contributions

Conceived and designed the experiments: NGA HA LS AS JD FG. Performed the experiments: NGA HA EMH MPP LS. Analyzed the data: NGA HA JD FG. Contributed reagents/materials/analysis tools: NGA HA EMH MPP LS AS JD FG. Wrote the paper: NGA HA JD FG.

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Paper II

Cerebellar ataxia and glutamic acid decarboxylase antibodies: immunologic profile and long-term effect of immunotherapy.

Ariño H, Gresa-Arribas N, Blanco Y, Martínez-Hernández E, Sabater L, Petit-Pedrol M, Rouco I, Bataller L, Dalmau JO, Saiz A, Graus F. JAMA Neurol. 2014 Aug;71(8):1009-16.

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Contribution:

We investigated the largest cohort of patients with this GAD-associated cerebellar ataxia and found that prompt immunotherapy, among other factors, is related to a better prognosis. We provide also clinical clues to recognize these patients.

Original Investigation

Cerebellar Ataxia and Glutamic Acid Decarboxylase Antibodies

Immunologic Profile and Long-term Effect of Immunotherapy

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IMPORTANCE Current clinical and immunologic knowledge on cerebellar ataxia (CA) with glutamic acid decarboxylase 65 antibodies (GAD65-Abs) is based on case reports and small series with short-term follow-up data.

OBJECTIVE To report the symptoms, additional antibodies, prognostic factors, and long-term outcomes in a cohort of patients with CA and GAD65-Abs.

DESIGN, SETTING, AND PARTICIPANTS Retrospective cohort study and laboratory investigations at a center for autoimmune neurologic disorders among 34 patients with CA and GAD65-Abs, including 25 with long-term follow-up data (median, 5.4 years; interquartile range, 3.1-10.3 years).

MAIN OUTCOMES AND MEASURES Analysis of clinicoimmunologic features and predictors of response to immunotherapy. Immunochemistry on rat brain, cultured neurons, and human embryonic kidney cells expressing GAD65, GAD67, α1-subunit of the glycine receptor, and a repertoire of known cell surface autoantigens were used to identify additional antibodies. Twenty-eight patients with stiff person syndrome and GAD65-Abs served as controls.

RESULTS The median age of patients was 58 years (range, 33-80 years); 28 of 34 patients (82%) were women. Nine patients (26%) reported episodes of brainstem and cerebellar dysfunction or persistent vertigo several months before developing CA. The clinical presentation was subacute during a period of weeks in 13 patients (38%). Nine patients (26%) had coexisting stiff person syndrome symptoms. Systemic organ-specific autoimmunities (type 1 diabetes mellitus and others) were present in 29 patients (85%). Twenty of 25 patients with long-term follow-up data received immunotherapy (intravenous immunoglobulin in 10 and corticosteroids and intravenous immunoglobulin or other immunosuppressors in 10), and 7 of them (35%) improved. Predictors of clinical response included subacute onset of CA (odds ratio [OR], 0.50; 95% CI, 0.25-0.99; $P = .047$) and prompt immunotherapy (OR, 0.98; 95% CI, 0.96-0.99; $P = .01$). Similar frequencies of serum GAD67-Abs were found in patients with CA (24 of 34 patients [71%]) and in patients with stiff person syndrome (20 of 28 patients [71%]). However, GAD67-Abs were found in all of the cerebrospinal fluid samples examined (22 samples from patients with CA and 17 samples from patients with stiff person syndrome). Glycine receptor antibodies but not other cell surface antibodies were identified in 4 patients with CA. The presence of glycine receptor antibodies did not correlate with any specific clinical feature.

CONCLUSIONS AND RELEVANCE In patients with CA and GAD65-Abs, subacute onset of symptoms and prompt immunotherapy are associated with good outcome. Persistent vertigo or brainstem and cerebellar episodes can herald CA and should lead to GAD65-Ab testing, particularly in patients with systemic organ-specific autoimmunities.

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Autoimmunity is increasingly recognized as a cause of cerebellar dysfunction. Cerebellar ataxia (CA), associated with antibodies against the 65-kDa isoform of glutamic acid decarboxylase (GAD65-Abs), is one of the best-characterized cerebellar syndromes in which autoimmune mechanisms probably have a relevant pathogenic role.[1] After stiff person syndrome (SPS), CA is the second most frequent neurologic disorder associated with GAD65-Abs.[2] Current knowledge of the clinical and immunologic profile of CA with GAD65-Abs is based on small series and single case reports, and the long-term outcomes after immunotherapy are unknown.[1,3]

The association of GAD65-Abs with various syndromes such as SPS or CA has led to several hypotheses regarding the possible pathogenic role of these antibodies. The results of experimental investigations using monoclonal antibodies against different GAD65 epitopes suggest that the various neurologic syndromes could be related to the pattern of epitope recognition by human serum.[4] Alternatively, the occurrence of CA or SPS could result from additional mechanisms mediated by T cells[5] or antibodies against surface antigens such as those described in several autoimmune encephalitis.[6] In this regard, γ-aminobutyric acid B receptor and glycine receptor antibodies (GlyR-Abs) have been described in patients with concurrent GAD65-Abs and limbic encephalitis or SPS.[7,8]

In this study, we retrospectively examined a cohort of patients with CA associated with GAD65-Abs. We aimed to better characterize the clinical presentation, the immunologic profile, the presence of additional antibodies against cell surface antigens, and the long-term response to immunotherapy.

Methods

Patients

The study was approved by the ethics committee of the Hospital Clinic, Barcelona, Spain. Written informed consent was obtained from all patients for the storage and use of serum and cerebrospinal fluid (CSF) samples for research purposes. Patients with CA and GAD65-Abs who were seen at the Hospital Clinic or whose serum or CSF samples were examined at the Institut d'Investigacions Biomèdiques August Pi i Suny, Barcelona, Spain, between December 15, 1994, and April 4, 2013, were included in the study if they met the following criteria: (1) predominant or isolated cerebellar dysfunction at presentation and the absence of another cause that could explain their CA, (2) available clinical information, and (3) the presence of high serum titers of GAD65-Abs confirmed by radioimmunoassay and immunohistochemistry.[3] Evidence has shown that positive serum immunoreactivity using rat cerebellar sections is associated with high GAD65-Ab levels on radioimmunoassay (usually >2000 U/mL).[2] Overall, 49 potential study patients were identified, 15 of whom were excluded because of a lack of clinical information. Long-term follow-up data were obtained in 25 of 34 patients (74%) (median, 5.4 years; interquartile range, 3.1-10.3 years). Data were obtained from clinical records, and information was collected from referring neurologists using a structured questionnaire mainly focused on the clinical presentation, the presence of neurologic symptoms preceding the cerebellar syndrome, concomitant symptoms of rigidity and spasms, and the response to immunotherapy. The onset of CA was defined as subacute when the cerebellar symptoms reached their nadir or required neurologic assessment within the first 3 months of symptom presentation. Ten patients were examined and followed up by 1 or more of us. Neurologic disability was measured by the modified Rankin Scale (mRS).[9] Patients with a history of cancer who met the criteria for definite or possible paraneoplastic neurologic syndrome were excluded from the study.[10] Serum and CSF samples used in the study were deposited in the Neuroinmunología collection of biological samples registered in the biobank of the Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain.

Autoantibody Assays

All laboratory techniques have been previously reported and are described in detail in the eMethods in the Supplement. These include immunohistochemistry on frozen rat brain, immunoblot of human GAD65 recombinant protein (Diamyd),[3] immunocytochemistry on cultures of rat hippocampal and cerebellar granular neurons,[3,11,12] and immunocytochemistry on human embryonic kidney 293 cells transfected with the α-1 subunit of the GlyR (obtained by gift) and with GAD65 and GAD67 (OriGene).[8]

Statistical Analysis

Nonparametric tests were used when the distribution of the analyzed variables differed from normal using the Kolmogorov-Smirnov test. Good outcome was defined as an mRS score of less than 3 at the last follow-up visit, and bad outcome was defined as an mRS score of 3 or higher. In treated patients, improvement was defined as a decrease of at least 1 point on the mRS at the last follow-up visit compared with the score at diagnosis. For the multivariate analysis, a generalized linear model[13] was used that included relevant or significant ($P \leq .10$ on univariate analysis) factors. As a dependent variable to analyze response to treatment, we used the change in mRS scores from the onset to the last follow-up visit. Odds ratios (95% CIs) were used to measure the effect of predictors on the exponential function of the regression coefficient. Statistical software (SPSS Statistics version 19; IBM) was used for the analyses.

Results

Clinical Characteristics at Diagnosis

The median age of patients was 58 years (range, 33-80 years); 28 of 34 patients (82%) were women. Twenty-nine patients (85%) had concomitant systemic organ-specific autoimmune disorders. Type 1 diabetes mellitus was present at diagnosis in 13 patients (38%), and the incidence increased to 17 of 25 patients (68%) during the follow-up period. Twenty-one patients (62%) had other organ-specific autoimmune disorders, mainly thyroiditis and pernicious anemia (**Table 1**). Thirteen patients (38%) had subacute presentation of CA lasting for weeks, while the other 21 patients (62%) had a chronic course progressing during months or years. Overall, the demo-

Table 1. Clinical and Immunologic Features of Patients Having CA or SPS With GAD65-Abs

Variable	CA (n = 34)	SPS (n = 28)	*P* Value
Age, median (range), y	58 (33-80)	56 (19-77)	.19
Female sex, No. (%)	28 (82)	26 (93)	.20
Type 1 diabetes mellitus at diagnosis, No. (%)	13 (38)	14 (50)	.25
Other organ-specific autoimmune disorder			
No. (%)	21 (62)	13 (46)	.17
Thyroiditis, No.	18	7	NA
Pernicious anemia, No.	7	3	NA
Vitiligo, No.	2	3	NA
Other, No.[a]	3	0	NA
Tumor, No.[b]	4	1	.36
Epilepsy, No. (%)	4 (12)	3 (11)	.61
GAD65-Ab radioimmunoassay, median (interquartile range), 1000 U/mL	20.8 (9.5-44.6)	15.0 (5.7-28.5)	.31
CSF oligoclonal bands, No./total No. (%)	16/22 (73)	5/17 (29)	.07
GAD65-Ab intrathecal synthesis, No./total No. (%)[c]	13/15 (87)	9/11 (82)	.57
X-fold of GAD65-Ab intrathecal production, median (range)[d]	8.9 (2.2-95.2)	5.3 (1.1-20.0)	.43
GAD67-Abs			
Serum, No. (%)	24 (71)	20 (71)	.45
CSF, No./total No. (%)[e]	22/22 (100)	17/17 (100)	NA

Abbreviations: CA, cerebellar ataxia; CSF, cerebrospinal fluid; GAD65-Abs, glutamic acid decarboxylase 65 antibodies; NA, not applicable; SPS, stiff person syndrome.

[a] Includes celiac disease, psoriasis, and myasthenia.

[b] Three tumors (thymoma, endometrial carcinoma, and breast cancer) diagnosed at least 7 years (range, 7-7.6 years) before the onset of their CA. The fourth patient manifested a myelodysplastic syndrome 6 years after the diagnosis of CA. The only tumor in patients with SPS was a case of breast cancer diagnosed 20 years after the onset of SPS.

[c] The index for intrathecal synthesis of GAD-Abs was calculated using the following formula: (CSF GAD-Ab Titer Divided by Serum GAD-Ab Titer) Divided by (CSF Albumin Level in Milligrams per Liter Divided by Serum Albumin Level in Milligrams per Liter).[2]

[d] Calculated in reference to the general IgG ratio instead of the albumin ratio in 13 patients with CA and 11 patients with SPS.[2]

[e] Seven patients having CA and 4 patients having SPS with CSF GAD67-Abs did not manifest these antibodies in serum.

graphic features and autoimmune clinical associations of 34 patients with CA were similar to those of 28 patients with SPS and GAD65-Abs.

Gait ataxia was the most common clinical presentation (31 patients), followed by limb ataxia (25 patients) that was asymmetric in 20, dysarthria (24 patients), and nystagmus (20 patients). Muscle rigidity and spasms were identified in 9 patients (26%), and 4 of them manifested electromyographic features of SPS (**Table 2**). Muscle rigidity and spasms occurred at the time of their CA in 5 patients and 2 to 5 years later in 3 patients. In 1 patient, leg spasms triggered by emotional stimuli or anxiety were present 2 years before the onset of her CA. Four patients also had epilepsy. In 3 of them, their epilepsy antedated the diagnosis of CA by 15, 13, and 2 years. Two of these patients met the criteria for refractory temporal lobe epilepsy. The fourth patient developed 2 generalized seizures 18 months after the onset of CA.

Neurologic symptoms antedating the diagnosis of CA were reported in 9 patients (26%), with 2 different profiles. Six patients reported fluctuating vertigo 7 to 26 months before developing CA. All manifested exacerbations that lasted days to weeks. During this period, the neurologic examinations showed no signs of CA. The remaining 3 patients had at least 1 episode of transient neurologic deficit, suggesting brainstem or cerebellar involvement, 2 to 24 months before the diagnosis of CA. The first patient had an episode of isolated vertical diplopia that lasted 10 days. The second patient had 2 episodes of dysarthria and gait ataxia that lasted a few days. The third patient developed dysarthria and right arm ataxia that lasted for 2 months and resolved spontaneously. The presence of prodromal symptoms was 3 times more common in men ($P = .07$).

Long-term Outcomes

Long-term follow-up data were available for 25 patients. **Figure 1** shows the type and duration of treatment during the course of the disease. Five patients received no immunotherapy, and 2 patients were untreated for more than 2 years. None of them improved, and the condition in 3 patients slowly deteriorated. Twenty patients received immunotherapy, which in 10 patients included intravenous immunoglobulin (IVIg), while 9 patients received intravenous methylprednisolone alone (4 patients) or in combination with IVIg (4 patients) or rituximab (1 patient). One patient was treated with oral prednisone (1 mg/kg/d). Among treated patients, 17 received various types of maintenance therapy: 6 were treated with IVIg during a median of 56.2 months (interquartile range, 24.4-121.5 months), and 11 received 1 or more regimen of oral corticosteroids, azathioprine, or mycophenolate mofetil.

The eFigure in the Supplement shows the degree of disability at diagnosis, at the end of the first treatment (up to 6 months), and at the last follow-up visit. At the last follow-up visit, 11 patients had an mRS score of less than 3 (good out-

Table 2. Symptoms of SPS in 9 Patients With CA and GAD65-Abs Having Muscle Rigidity and Spasms

Sex/Age at Diagnosis, y	Distribution of Stiffness	Other SPS Symptoms	Neurophysiological Findings	Temporal Relationship With CA	Treatment	Clinical Course of SPS and CA
F/53	Legs	Frequent falls	Compatible with SPS[a]	Same time	IVIg	No change with SPS and CA
F/77	Legs, trunk	Leg spasms, lumbar pain	Compatible with SPS	2 y Later	Oral IS, rituximab	SPS improved and later relapsed, CA improved and then progressed
F/76	Right leg	Leg spasms	Compatible with SPS	Same time	IVIg, rituximab, chronic IS	Remission of SPS after first-line treatment and relapsing at last visit (5 y), slow partial improvement of CA
F/50	Legs first, then trunk	Leg spasms, lumbar hyperlordosis	Compatible with SPS	2 y Later	PE, oral IS, rituximab	SPS improved,CA stable
M/74	Leg, trunk	Leg spasms	Sensorimotor polyneuropathy	Same time	IVIg, oral IS	Improvement of both syndromes after 1 y
F/40	Right leg	Leg spasms	Signs of denervation	Same time	IVIg, oral IS	Both syndromes stable
F/59	Legs, trunk	Lumbar pain, hyperlordosis	Normal	Same time	IVIg, oral IS	No response after 4 mo, patient lost to follow-up data
F/52	Legs, first asymmetric	Leg spasms	Not done	5 y Later	IVIg, PE	Mild improvement of SPS spasms, CA stable
F/52	Legs	Leg spasms, agoraphobia	Normal under benzodiazepine treatment	2 y Before[b]	IVIg	Clear improvement of SPS after 1 cycle, no CA immediate effect, patient lost to follow-up data

Abbreviations: CA, cerebellar ataxia; F, female; GAD65-Abs, glutamic acid decarboxylase 65 antibodies; IS, immunosuppression; IVIg, intravenous immunoglobulin; M, male; PE, plasma exchange; SPS, stiff person syndrome.

[a] Continuous motor unit potentials firing at rest and during contraction of antagonist muscles in needle electromyographic recordings.

[b] The patient experienced leg spasms triggered by emotional stimuli and prominent anxiety. She was diagnosed as having conversion disorder until she developed CA.

come), and 14 patients had an mRS score of 3 or higher (bad outcome). Patients with good outcome had a better mRS score at diagnosis than patients with bad outcome (median, 2 vs 3; $P = .01$) and responded to immunotherapy more frequently (6 patients [55%] vs 1 patient [7%], $P = .01$) (**Table 3**).

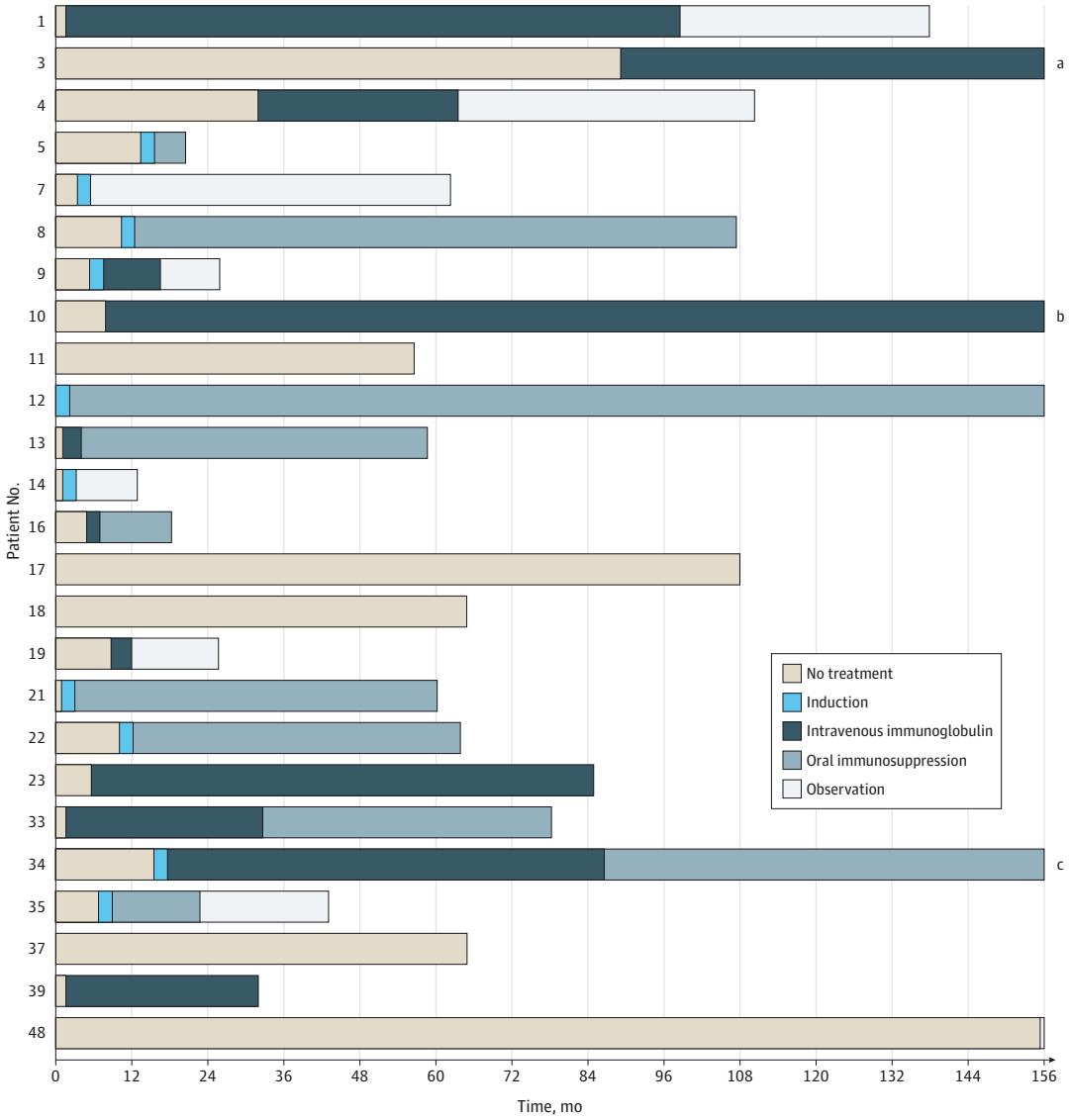
Among 20 patients who were treated, 10 showed clinical improvement (≥1 point on the mRS during the first 6 months of treatment), which in 7 patients (35%) persisted at the last follow-up visit. Four of them were treated with pulses of intravenous methylprednisolone, and 3 were treated with IVIg. Four patients also received maintenance immunotherapy (for 1.1-4.9 years) with oral immunosuppressors (3 patients) or IVIg (1 patient). Improvement after immunotherapy was more common in patients with subacute onset (5 of 7 subacute patients [71%] vs 2 of 13 chronic patients [15%], $P = .02$). Age, sex, mRS score at diagnosis, the presence of type 1 diabetes mellitus or other autoimmune disorders, and evidence of cerebellar atrophy on magnetic resonance imaging at diagnosis were similar in responders and nonresponders. On multivariate analysis, favorable predictors of clinical response included subacute onset of CA (odds ratio, 0.50; 95% CI, 0.25-0.99; $P = .047$) and prompt immunotherapy (odds ratio, 0.98; 95% CI, 0.96-0.99; $P = .01$).

Immunologic Studies

All serum and CSF samples from patients with CA and SPS included in the study showed reactivity to human embryonic kidney cells transfected with GAD65. Similarly, all but one CA serum samples were positive in immunoblots of GAD65 recombinant protein, suggesting that the antibodies from patients with CA and SPS recognize linear epitopes. The assessment of GAD65-Abs by immunohistochemistry revealed that in all cases the predominant IgG isotype was IgG1. Additional isotypes were detected in a small proportion of patients, including IgG3 in 2 of 34 patients with CA and 2 of 28 patients with SPS and IgG2 in 2 of 34 patients with CA and 6 of 28 patients with SPS.

The frequencies of serum GAD67-Abs were similar in patients with CA (24 of 34 patients [71%]) and in patients with SPS (20 of 28 patients [71%]). GAD67-Abs were found in all CSF samples available from 22 patients with CA and from 17 patients with SPS. GAD67-Abs were present in CSF samples but not in serum samples of 7 patients with CA and 4 patients with SPS having paired serum and CSF samples (**Figure 2**). Serum and CSF samples from patients with CA did not immunoreact with live hippocampal and granular cerebellar neurons, and none manifested antibodies against *N*-methyl-D-aspartate, γ-aminobutyric acid B, and α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors or leucine-rich glioma-inactivated protein 1, dipeptidyl peptidase-like protein 6, and contactin-associated protein 2 antigens. In contrast, 4 patients with CA had GlyR-Abs in serum samples but not in the 2 CSF samples available. The presence of GlyR-Abs did not correlate with any specific clinical feature, no patient

Figure 1. Individual Therapy Regimens in 25 Patients With Long-term Follow-up Data



For visual purposes, periods of treatment or between-periods are approximate, and therapeutic schemes are simplified. See the Long-term Outcomes subsection of the Results section for a description of the types of induction therapy and oral immunosuppression.

[a]Total follow-up: 283 months. Beyond 156 months, patient 3 was treated with oral immunosuppression for 63 months and then observation.

[b]Total follow-up: 209 months. Beyond 156 months, patient 10 continued treatment with intravenous immunoglobulins.

[c]Total follow-up: 197 months. Beyond 156 months, patient 34 continued treatment with oral immunosuppression.

had associated symptoms of SPS, and only one patient responded to immunotherapy.

Discussion

To our knowledge, this is the first study to describe the long-term outcomes in a series of patients with CA and GAD65-Abs. Our findings indicate that patients with subacute presentation of CA are more likely to respond to immunotherapy and achieve good functional status (mRS score, <3) and confirm that a shorter delay in the initiation of immunotherapy predicts clinical improvement.[14]

Previous autopsy studies[15,16] of patients with CA and GAD65-Abs revealed selective loss of Purkinje cells. However, the clinical improvement observed in some of our patients indicates that part of the cerebellar dysfunction at the time of diagnosis may be due to functional impairment that can be reversed by early onset of immunotherapy. Although the pathogenic role of GAD65-Abs is unclear, studies[17,18] have shown that they interfere with the γ-aminobutyric acid-ergic synaptic transmission in tissue culture systems and that these effects are reversible after removing the GAD65-Abs. In addition, intracerebellar injection of GAD65-Abs induces an increase in glutamate levels that may lead to glutamate excitotoxic effects.[4] These neurochemical and neurophysiological

Table 3. Clinical Features of 25 Patients With Long-term Follow-up Data by Good Outcome (mRS Score, <3) and Bad Outcome (mRS Score, ≥3)

Variable	Good Outcome (n = 11)	Bad Outcome (n = 14)	*P* Value
Age, median (range), y	69 (33-76)	54 (36-79)	.58
Female sex, No.	9	13	.41
Type 1 diabetes mellitus at diagnosis, No.	7	10	.50
Associated SPS or epilepsy, No.	5	5	.46
Subacute onset, No.	6	3	.09
mRS score at diagnosis, median (range)	2 (1-4)	3 (2-5)	.01
GAD65-Ab radioimmunoassay, median (interquartile range), 1000 U/mL	11.5 (6.6-20.8)	20.1 (9.3-31.5)	.28
Time to treatment, median (range), mo[a]	5.88 (0.99-64.95)	6.70 (0.13-165.40)	.49
Treated, No.	9	11	.62
Induction therapy[b]	3	5	.49
Maintenance therapy	7	10	.50
IVIg as chronic therapy[c]	3	4	.64
Improvement after therapy[d]	6	1	.01
Total duration of disease, median (range), y	5.02 (1.52-9.15)	8.01 (0.98-23.61)	.08

Abbreviations: GAD65-Ab, glutamic acid decarboxylase 65 antibody; IVIg, intravenous immunoglobulin; mRS, modified Rankin Scale; SPS, stiff person syndrome.

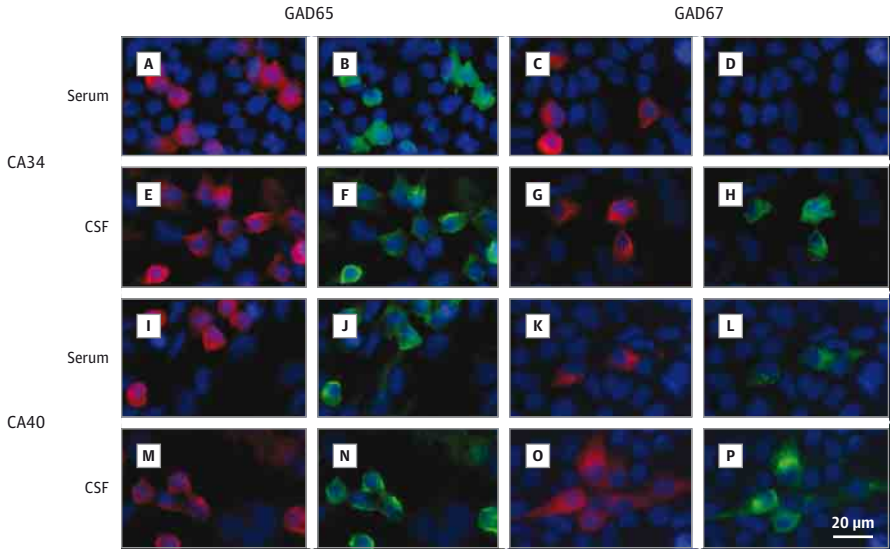
[a] From the onset of symptoms to immunotherapy.

[b] Includes high dose of corticosteroids, rituximab, or cyclophosphamide.

[c] The other patients received immunossupressors (corticosteroids, azathioprine, or mycophenolate mofetil).

[d] Defined as sustained decrease in mRS score of at least 1 point in treated patients at the last follow-up visit compared with mRS score at diagnosis.

Figure 2. Reactivity of Serum and Cerebrospinal Fluid (CSF) Samples to Fixed Human Embryonic Kidney Cells Expressing Glutamic Acid Decarboxylase 65 (GAD65) and GAD67



Shown is reactivity of fixed cells expressing human GAD65 and GAD67 to serum samples (A-D and I-L) and CSF samples (E-H and M-P) in 2 patients with cerebellar ataxia and GAD65 antibodies (green) and to a commercial monoclonal antibody against GAD65 or GAD67 (red). The nuclei of the cells are stained with 4′,6-diamidino-2-phenylindole. Patient CA34 manifests GAD67 antibodies in CSF (H) but not in serum (D).

abnormalities could initially affect the function of Purkinje cells without causing irreversible damage but may cause their death in the long run.

The retrospective design of this study and the multicenter locations of the patients make it difficult to recommend a particular type of immunotherapy. The effect of various immunotherapies has been described in single case reports (summarized in the eTable in the Supplement).[19-29] As in the present series, the most common treatments used were cycles of IVIg or methylprednisolone. Most of the described patients had a chronic presentation of symptoms, and treatment was started after a median delay of 12 months (range, 2-120 months). Improvement was reported in 13 of 16 patients (81%) compared with 7 of 20 patients (35%) in this series. However, the figures are not comparable because the degree of improvement reported in many of these case reports would not fulfill the required decrease of at least 1 point on the mRS as used herein (eTable in the Supplement).[18-28]

As in SPS,[30,31] we observed that patients with CA who responded to immunotherapy did so early, during the first 6 months. A lack of improvement in this short period should be an indication for switching to a second-line immunotherapy or stopping treatment.[32] Our study does not clarify whether maintenance immunotherapy is useful: 3 of 7 patients who improved with the initial treatment remained stable without subsequent immunotherapy.

We have identified a previously unrecognized feature to date of patients with CA and GAD65-Abs. Three patients (9%)

reported episodes of diplopia or combinations of dysarthria and ataxia of unclear etiology months before the development of full-blown CA. In addition, 6 patients (18%) reported isolated vertigo in the absence of other symptoms of cerebellar dysfunction. We consider that vertigo in these patients was heralding the development of more widespread involvement of the cerebellum. This feature has been observed in other cerebellar syndromes. In patients with spinocerebellar ataxia, episodic vertigo antedated the development of gait ataxia by several years in 4% of patients.[33] Investigations of selective ischemic infarcts of the cerebellum indicate that patients who were seen with isolated vertigo more frequently had the infarct in the caudal vermis.[34] Taken together, these data suggest that in some patients GAD65 autoimmunity may result in subtle, focal, or transient symptoms, likely representing involvement of selective brainstem or cerebellar regions. In some patients, particularly those with diseases that are associated with GAD autoimmunity (eg, type 1 diabetes mellitus), the development of persistent vertigo of unknown etiology or episodes of diplopia, dysarthria, or ataxia, should lead to GAD65-Ab testing. In that clinical scenario, the detection of high-titer GAD65-Abs (usually >2000 U/mL) should raise concern about impending CA.

In our patients with CA, the immunologic response against GAD did not differ from that observed in patients with SPS. We found no significant increase in intrathecal production of GAD65-Abs in patients having CA compared with those having SPS, as previously suggested.[35] A relevant observation was that all patients in whom CSF samples could be analyzed manifested GAD67-Abs, despite that some of them did not have these antibodies in serum samples. The presence of GAD67-Abs only in CSF, along with previous demonstration of intrathecal synthesis of GAD65-Abs[35] and a different epitope repertoire noted between paired serum and CSF samples,[36] strongly supports the presence of GAD-specific B cells in the central nervous system and emphasizes the importance of examining the CSF in autoimmune disorders of the central nervous system.

Except for 4 patients with CA who had concomitant GlyR-Abs in serum samples, we did not find (as suggested in SPS[37]) other antibodies against neuronal surface antigens. Glycine receptor antibodies were initially described in patients having progressive encephalomyelitis with rigidity and myoclonus.[38] More recently, GlyR-Abs were reported in 12% to 15% of patients with SPS (with or without GAD65-Abs) and in 3% of patients with epilepsy.[8,39,40] In our patients, the significance of GlyR-Abs is unknown because the clinical course and outcome did not differ in patients without this antibody. In 2 of these 4 patients, CSF samples were available and were negative for GlyR-Abs in both of them. Although earlier described patients having progressive encephalomyelitis with rigidity and myoclonus showed GlyR-Abs in serum and CSF samples,[41,42] in patients with SPS or epilepsy GlyR-Abs were usually studied based on serum samples only.[8,39,40] Therefore, it is unclear whether the presence or absence of GlyR-Abs in CSF is associated with different clinical phenotypes as recently reported in other autoimmune encephalitis.[43] Future studies should determine the degree of syndrome specificity of GlyR-Abs, comparing paired serum and CSF samples in larger groups of patients and control subjects.

Conclusions

Our findings reveal that patients with CA and GAD65-Abs may respond to immunotherapy and maintain good functional status for many years. The retrospective design of the study prevents our making definite recommendations, but in our experience the use of IVIg or corticosteroids should be considered in all patients with CA and GAD65-Abs, particularly those with subacute presentation. Early initiation of treatment likely offers a greater chance of improvement.

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Acquisition, analysis, or interpretation of data: All authors.
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Supplementary Online Content

Ariño H, Gresa-Arribas N, Blanco Y, et al. Cerebellar ataxia and glutamic acid decarboxylase antibodies: immunologic profile and long-term effect of immunotherapy. *JAMA Neurology*. Published online June 16, 2014. doi:10.1001/jamaneurol.2014.1011.

This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods. Supplemental Methods

1. *GAD65-ab radioimmnunoassay*.

GAD65-ab were measured by radioimmunoassay using a commercial kit (CIS biointernational. France) following the manufacturer's instructions. Briefly, 20 µl of standards (SMS serum containing GAD-Ab at different dilutions expressed in arbitrary units U/ml), and serum samples, were incubated with 50 µl of ^{125}I-labeled human recombinant GAD65 for 2 hours at room temperature. Then, 50 µl of protein A-sepharose was added, and the mixture incubated for 1 hour at room temperature. After centrifugation at 1500 g for 30 minutes at 4ºC, the precipitates were counted for ^{125}I with a gamma scintillation counter. The results were interpolated in the standard curve constructed using the dilutions of the positive control serum. For each serum conditions were established in that appropriate dilutions of the serum produced results that were in the straight line portion of the standard curve.

2. *Immunohistochemistry*.

Wistar rats were anesthetized and perfused with saline followed with 4% paraformaldehyde in phosphate buffered saline (PBS). The cerebellum was further fixated with 4% paraformaldehyde for 4 hours and cryoprotected with 20% sucrose in PBS overnight. Samples were frozen in isopentane chilled in liquid nitrogen. 10 mm frozen sections were air-dried for 30 minutes and, after inhibition of endogenous peroxidase with 0.3% hydrogen peroxide in PBS for ten minutes, were sequentially incubated with 10% normal goat serum for 20 minutes, patient's serum (screening dilution 1:500) for 3 hours at 37°C, biotinylated goat anti-human IgG for 30 minutes, and the avidin biotin immunoperoxidase complex (Vector Laboratories, Burlingame, CA) for 30 minutes. The reaction was developed with 0.05% diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St.Louis) with 0.01% hydrogen peroxide in PBS with 0.5% Triton X-100. Dilution of antibodies was done in PBS with 0.3% Triton X-100. To study the GAD65-ab IgG subtypes, brain section incubated with the positive GAD65-ab serum were subsequently incubated with secondary biotinylated mouse monoclonal anti-human IgG 1-4 (Sigma-Aldrich, St.Louis) diluted 1:100 for IgG1 and 1:200 IgG2-4.

3. *GAD65 immunoblot*.

Human GAD65 recombinant protein (Diamyd, Stockholm, Sweden) was electrophoretically separated in a 4-12% Bis-Tris polyacrylamide gel (Life Technologies, Carlsbad, CA) and transferred to PVDF membrane. After blocking with 5% dry Carnation milk, strips were incubated with the patient's serum (1:1000 dilution) or GAD-6 monoclonal antibody (Hybridoma Bank, Iowa City, IA) overnight at room temperature and incubated with biotinylated goat anti-human IgG, diluted 1:1,000, or horse antimouse IgG (Vector Laboratories, Burlingame, CA), diluted 1:10000 in 5% normal goat serum for 1 hour. Strips were then immunoreacted with an avidin-biotin technique and developed with diaminobenzidine tetrahyrochloride.

4. *Immunocytochemistry on HEK293 cells.*

Fixed cells

HEK293 cells were transfected with plasmids containing the human GAD65 or GAD67 (OriGene, Rockville, MD). Cells were grown for 24 hours after transfection before assessment. Transfected cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 and then incubated with patients' serum (1:40) or CSF (1:5) along with a commercial mouse antibody against against the GAD65 (1:4000, GAD-6 Hybridoma Bank, Iowa City, IA), or the GAD67 (1:4000, Abcam, Cambridge), for 1 hour at room temperature, and the corresponding fluorescent secondary antibodies (Alexa Fluor 488 goat anti-human IgG, A11013, diluted 1:1000; and Alexa Fluor 594 goat anti-mouse IgG, A11032, diluted 1:1000, both from Life Technologies, Carlsbad, CA). Results were photographed under a fluorescence microscope using Zeiss Axiovision software.

Live cells

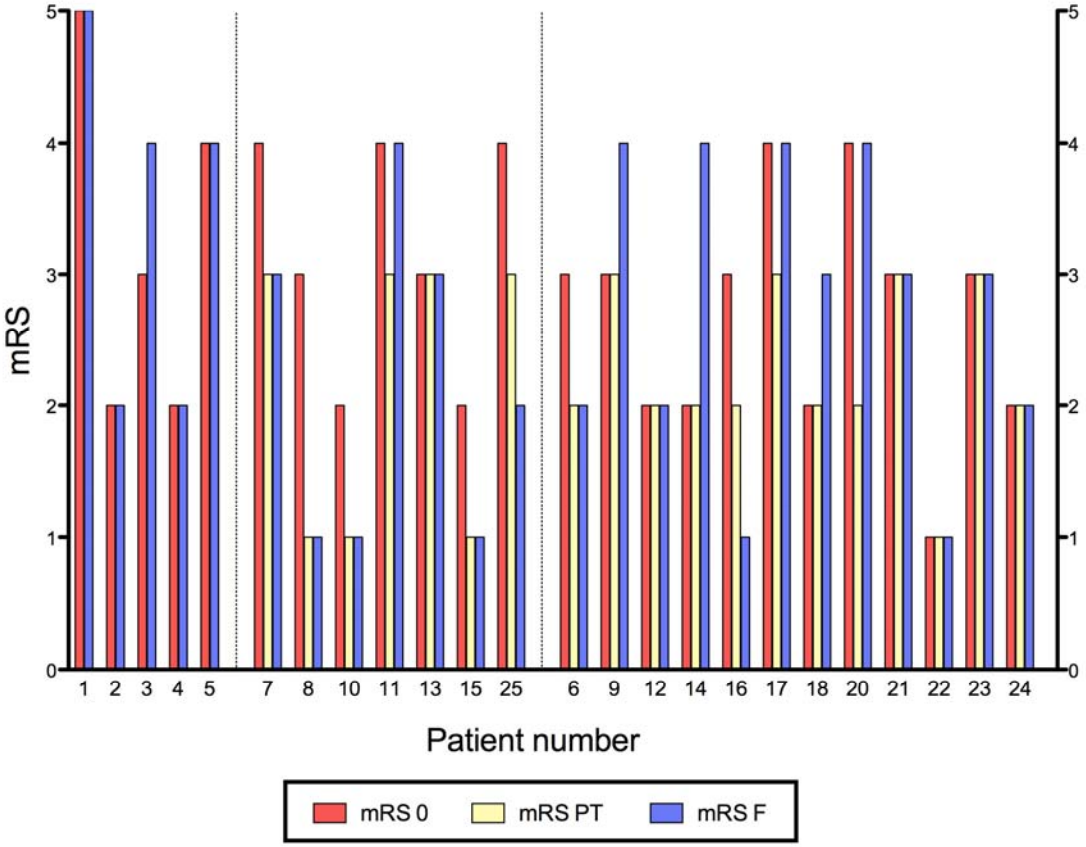
HEK293 cells were transfected with plasmids containing the human α1 subunit of the glycine receptor. Live transfected HEK cells were incubated with serum (1:40) or CSF (1:5) of the patient together with the commercial antibody against α1 subunit of the glycine receptor (dilution 1:1000, Synaptic Systems Goettingen, Germany) for 1

hour at 37ºC, washed, and fixed with 4% paraformaldehyde for 5 minutes. After washing cells were then incubated with the corresponding Alexa Fluor secondary antibodies indicated above.

5. *Primary neuronal cell cultures and in vivo immunocytochemistry.*

Hippocampal neurons were obtained from E18 Wistar rat embryos. Cells were enzymatically and mechanically disrupted and resuspended in Neurobasal medium supplemented with B27 (Life Technologies, Carlsbad, CA).[1] Granular cell cultures were prepared from dissected cerebella of 8-day-old Wistar rats and processed equally as hippocampal neurons but using Neurobasal-A medium containing 25 mM of KCl and supplemented with B27.[2] Cells were plated in poly-L-lysine pre-coated P24 plates and 10 µM cytosine-D-arabinofuranoside (Sigma-Aldrich, St. Louis, MO) was added to the cultures 20 h after plating, to prevent proliferation of non neuronal cells.
In the immunocythochemistry experiments, samples (1:5 CSF dilution and 1:200 serum dilution) were incubated on live neurons for 1 hour, then fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton TX-100. Appropriate fluorescent secondary antibodies were applied and the coverslips were mounted with Vectashield with DAPI mounting media (Vector Laboratories, Burlingame, CA) and visualized with an Axio Imager M2 ZEISS microscope.

eFigure. Degree of Disability at Diagnosis, at the End of the First Treatment (up to 6 Months), and at the Last Follow-up Visit



Individual outcomes in 25 patients with CA and GAD 65-ab. Bars represent the mRS at onset (mRS 0); at 6 months of treatment (mRS PT), and at last follow-up visit (mRS F). Patients are grouped in non-treated (patient 1 to 5) and treated with subacute (7 to 25) and chronic (6 to 24) onset. Patients' number are the same than in Figure 1.

eTable. Case Reports of Patients With Cerebellar Ataxia and GAD65-Ab Treated With Immunotherapy

Reference[a]	Age[b] /sex	Onset CA	Delay[c]	Induction	Maintenance	Outcome (time[d])
Virgilio[3]	76/M	chronic	7	IV steroids	Steroids	Improved (11)
Planche 1[4]	72/F	subacute	6	IVIg x2, rituximab	No	Progressed after IVIg, improved after rituximab* (10)
Planche 2[4]	73/F	unknown	36	IVIg, rituximab	IVIg x3	Improved* (13)
Planche 3[4]	65/M	unknown	36	IVIg x2, rituximab, Cy	No	Progressed (17)
Pedroso[5]	51/M	chronic	108	IVIg x3	No	Improved (4)
Nociti[6]	42/F	chronic	7	Steroids	AZA	Improved (21)
Nanri 1[7]	51/F	unknown	120	IVIg x4	No	Improved* (36)
Nanri 2[7]	72/F	unknown	48	IVIg	No	Improved* (12)
Lauria[8]	66/F	chronic	5	IV steroids, IVIg	Steroids, Cy	Improved (17)
Bonnan 2[9]	38/F	chronic	12	IV steroids, IVIg, PE	No	Improved* (36)
Bonnan 3[9]	45/F	chronic	12	IV steroids, IVIg	Periodic IVIg, AZA	Stable (31)
Bonnan 4[9]	75/F	chronic	10	IV steroids	No	Stable (12)
Bayreuther[10]	47/F	chronic	24	IVIg	No	Improved* (6)
Vulliemoz[11]	58/M	subacute	2	IV steroids	Steroids, AZA	Improved (8)
Abele[12]	50/F	chronic	216	IVIg x2	No	Improved* (2)
McFarland[13]	70/M	chronic	9	IVIg, PE	AZA	Improved (19)

[a]Same references indexed in main text, but in different order. [b]At diagnosis. [c]Months from onset of CA until first treatment. [d]Months from onset of treatment to last follow-up visit. *Description in text reflects mild improvement. None of them improved more than 12/100 points in the ICARS (International Cooperative Ataxia Rating Scale). These patients probably would not achieve a shift in the mRS and the effect of therapy should be interpreted as stable response when compared with the data of our series.

AZA: azathioprine; CA: cerebellar ataxia; Cy: cyclophosphamide; IV: intravenous; IVIg: intravenous immunoglobulins; PE: plasma exchange

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Paper III

Paraneoplastic Neurological Syndromes and Glutamic Acid Decarboxylase Antibodies.

Ariño H*, Höftberger R*, Gresa-Arribas N, Martínez-Hernández E, Armangue T, Kruer MC, Arpa J, Domingo J, Rojc B, Bataller L, Saiz A, Dalmau J, Graus F. JAMA Neurol. 2015;72(8):874-881.
(*These authors contributed equally to this article)

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Highlighted with an editorial

Contribution:
We identified predictive factors of cancer in patients with GAD-ab and neurological syndromes. The clinical presentation was the most important, but laboratory investigations may be helpful to increase the suspicion of cancer.

EDITORIAL

Association of Paraneoplastic Neurological Disorders With Glutamic Acid Decarboxylase Antibodies

Christian G. Bien, MD

An increasing number of antineural IgG antibodies have been detected since the 1980s in patients with autoimmune central nervous system disease. Antibodies to intracellular antigens were initially found and antibodies to antigens on the neural surface have now been identified. Antibodies to the intracellular enzyme glutamic acid decarboxylase (GAD) were one of the earliest antibodies discovered.[1] However, not all antibodies have the same value. This means that not all are pathogenic, specific for defined syndromes, and indicative for responsiveness to immunological treatment. Antibodies to the *N*-methyl-D-aspartate receptor and defined antigens in the voltage-gated potassium channel complex, such as leucine-rich glioma inactivated protein 1 and contactin-associated protein 2 (most likely the antibodies to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, the γ-aminobutyric acid B receptor, the glycine receptor, and others as well), are high-rank antibodies in that sense. However, other antibodies have their flaws because they lack 1 or more of these criteria. Antibodies to GAD are quite specific and are found in stiff-man syndrome, cerebellar ataxia, limbic encephalitis, and temporal lobe epilepsy.[2] Specificity beyond 1 single syndrome is not uncommon. Even the high-rank antibodies are not entirely specific, as observed in the *N*-methyl-D-aspartate receptor antibodies.[3,4] However, as already discussed in the Solimena et al article,[1] the intracellular location of GAD makes an in vivo immunological reaction unlikely; GAD antibodies are probably not pathogenic on their own.[1] The response of GAD antibodies to treatment is not as effective as, for example, antibodies to elements of the voltage-gated potassium channel complex.[5] Taken together, GAD antibodies, even if they are high titer, are somehow considered second-class antibodies.

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Despite these limitations, GAD antibodies have fascinating features. Neurological researchers have made observations that keep challenging the too-nihilistic view on the value of these antibodies. Glutamic acid decarboxylase antibodies are found in patients with neurological symptoms usually at high titers and can be picked up by a number of techniques, such as radioimmunoprecicitation or enzyme-linked immunosorbent assays, rodent brains, cell-based assays, and immunoblots. These antibodies are relatively frequent. Although they are directed to an intracellular target, they are not as regularly associated with paraneoplastic syndrome (PNS) as the classic onconeural antibodies that are also directed to intracellular antigens, such as Hu and Yo, among others.[2] Glutamic acid decarboxylase antibodies have prognostic implications because they usually indicate long-term difficult-to-treat courses. For example, epilepsy associated with GAD antibodies is usually resistant to antiepileptic and immunotherapeutical interventions.[6] However, in stiff-man syndrome with GAD antibodies, there is a prospective placebo-controlled immunotherapy trial demonstrating a superior outcome on intravenous immunoglobulins.[7] Glutamic acid decarboxylase autoimmunity often does not stand alone but is part of polyendocrine autoimmunity and is frequently associated with additional antibodies (eg, thyroid antibodies).[2] Glutamic acid decarboxylase is a prominent enzyme in the central nervous system. It catalyzes the conversion of the main excitatory neurotransmitter (glutamate) to the main inhibitory transmitter (γ-aminobutyric acid). Should that not suggest a potential pathogenic potency of the GAD antibodies, given the frequent hyperexcitatory characters of associated neurological syndromes? Several animal transfer studies have been performed with material from patients with GAD antibodies. These studies have been interpreted as showing pathogenic effects of the antibodies, possibly modulated by subtle differences in antigenic targeting (see the Manto et al study[8] with further references). However, other authors suggested that it is not the GAD antibodies but accompanying other antibodies that exert these pathogenic effects. An example for this position is a study by Gresa-Arribas et al[9] with Francesc Graus, MD, PhD, as the senior author. It is 1 of more than 20 studies on GAD antibodies that he has authored.

In this issue of *JAMA Neurology*, Ariño et al[10] present novel data on GAD antibodies. In their collection of 121 cases with high-titer GAD antibodies, the authors found an unexpectedly large number of patients (n = 15; 12%) with tumors classified as having a definite or possible PNS. None of them had classic onconeural antibodies. However, 8 of 15 patients with PNS had additional antibodies directed to the following targets: γ-aminobutyric acid B receptors, γ-aminobutyric acid A receptors, glycine receptors, or unknown targets on neuronal cell surfaces. This underlines the impression that GAD antibodies more likely come together with additional antibody reactivities compared with other antibodies. Although there have been some small-scale studies on patients with GAD antibodies and tumors, to our knowledge, this is the first large systematic study of this constellation. Ariño et al[10] are well known for their experience with PNS. This makes it possible that the large number of these syndromes in their biobank is owing to a higher likelihood that materials from patients with tumors are sent to their laboratory. However, even if one takes into ac-

count a slight overestimation of this constellation, the study is novel and important. Some years ago prior to the era of the surface antibodies, the clinical description of patients with PNS with GAD antibodies would have been possible, however, not with evidence of concomitant antibodies that might portend an underlying tumor. With the emergence of antibodies to surface antigens, half of patients with PNS and GAD antibodies are found to harbor such surface antibodies as well. Many neurologists conclude that a comprehensive antibody workup of patients, including those to the novel surface antigens, should be part of an adequate assessment of patients suspected to have an immunological basis of their central nervous system disease. One can imagine that the 121 patients from the Ariño et al study[10] would have only been tested for GAD antibodies and classic onconeural antibodies (as was typical several years ago). Detection of the usually nonparaneoplastic GAD antibodies often might have satisfied the physician prematurely. Part of the tumors might have gone unnoticed, with potentially negative consequences for the affected patients quoad vitam. However, with inclusion of the surface antigens in the antibody test battery, an additional antibody was found that would trigger a tumor search in at least half of the patients with PNS. For example, γ-aminobutyric acid B antibodies are known to occur in half of cases with tumors. However, because half of the patients with PNS and GAD antibodies had negative test results for other known antibodies, Ariño et al[10] suggested screening every patient with GAD antibodies for a potentially underlying tumor.

As a relevant further merit of the study, the authors refined our view of the syndromic presentation of patients with positive GAD antibodies. Ariño et al[10] observed in their own patients and those reported in the literature that paraneoplastic cases with GAD antibodies were particularly likely to have a classic PNS (eg, limbic encephalitis and subacute cerebellar degeneration) compared with less specific presentations, such as pure epilepsy or stiff-man syndrome. Another finding was that paraneoplastic patients with stiff-man syndrome and those with a thymic neoplasm seemed to have a particularly good outcome.

Glutamic acid decarboxylase antibodies are worth being studied as part of a broad antibody search in patients with suspected autoimmune or paraneoplastic neurological disorders. These antibodies may pave the way to the identification of a tumor. Even if a patient does not have a paraneoplastic syndrome, GAD antibodies have a sufficient syndromic specificity to terminate an extensive diagnostic process if found in a patient with a typical GAD antibody syndrome. Glutamic acid decarboxylase antibodies have prognostic implications. They often herald a chronic and difficult-to-control disorder without a rapid and terminal decline. These antibodies may occur in patients with underlying tumors; thus, a tumor search is warranted. By clarifying this, Ariño et al[10] have enhanced our understanding and refined our management of neurological conditions associated with GAD antibodies.

ARTICLE INFORMATION

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Conflict of Interest Disclosures: Dr Bien gave scientific advice to Eisai Inc and UCB Inc; undertook industry-funded travel with support of Eisai Inc, UCB Inc, Desitin, and Grifols; obtained honoraria for speaking engagements from Eisai Inc, UCB Inc, Desitin, DiaMed, and Fresenius Medical Care; and received research support from Astellas Pharma Inc, Octapharma, DiaMed, and Fresenius Medical Care. Dr Bien's employer (Krankenhaus Mara) runs a laboratory for the detection of autoantibodies, including those described in this article. External senders were charged for antibody diagnostics.

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Original Investigation

Paraneoplastic Neurological Syndromes and Glutamic Acid Decarboxylase Antibodies

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IMPORTANCE Little is known of glutamic acid decarboxylase antibodies (GAD-abs) in the paraneoplastic context. Clinical recognition of such cases will lead to prompt tumor diagnosis and appropriate treatment.

OBJECTIVE To report the clinical and immunological features of patients with paraneoplastic neurological syndromes (PNS) and GAD-abs.

DESIGN, SETTING, AND PARTICIPANTS Retrospective case series study and immunological investigations conducted in February 2014 in a center for autoimmune neurological disorders. Fifteen cases with GAD65-abs evaluated between 1995 and 2013 who fulfilled criteria of definite or possible PNS without concomitant onconeural antibodies were included in this study.

MAIN OUTCOMES AND MEASURES Analysis of the clinical records of 15 patients and review of 19 previously reported cases. Indirect immunofluorescence with rat hippocampal neuronal cultures and cell-based assays with known neuronal cell-surface antigens were used. One hundred six patients with GAD65-abs and no cancer served as control individuals.

RESULTS Eight of the 15 patients with cancer presented as classic paraneoplastic syndromes (5 limbic encephalitis, 1 paraneoplastic encephalomyelitis, 1 paraneoplastic cerebellar degeneration, and 1 opsoclonus-myoclonus syndrome). When compared with the 106 non-PNS cases, those with PNS were older (median age, 60 years vs 48 years; $P = .03$), more frequently male (60% vs 13%; $P < .001$), and had more often coexisting neuronal cell-surface antibodies, mainly against γ-aminobutyric acid receptors (53% vs 11%; $P < .001$). The tumors more frequently involved were lung (n = 6) and thymic neoplasms (n = 4). The risk for an underlying tumor was higher if the presentation was a classic PNS, if it was different from stiff-person syndrome or cerebellar ataxia (odds ratio, 10.5; 95% CI, 3.2-34.5), or if the patient had coexisting neuronal cell-surface antibodies (odds ratio, 6.8; 95% CI, 1.1-40.5). Compared with the current series, the 19 previously reported cases had more frequent stiff-person syndrome (74% vs 13%; $P = .001$) and better responses to treatment (79% vs 27%; $P = .005$). Predictors of improvement in the 34 patients (current and previously reported) included presentation with stiff-person syndrome and the presence of a thymic tumor.

CONCLUSIONS AND RELEVANCE Patients with GAD-abs must be screened for an underlying cancer if they have clinical presentations different from those typically associated with this autoimmunity or develop classic PNS. The risk for cancer increases with age, male sex, and the presence of coexisting neuronal cell-surface antibodies.

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Supplemental content at jamaneurology.com

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High serum levels of antibodies to the synaptic enzyme glutamic acid decarboxylase (GAD-abs) is a very sensitive biomarker of stiff-person syndrome (SPS) and have also been described in subgroups of patients with limbic encephalitis (LE),[1] cerebellar ataxia,[2] epilepsy, and isolated cases of palatal tremor, as well as downbeat or periodic alternating nystagmus.[3] Patients with neurological syndromes associated with GAD-abs are not considered at risk for cancer and extensive search for a tumor is not indicated unless they harbor additional onconeural antibodies. However, there are case reports of patients with GAD-abs whose cancer was identified by the time of the neurological diagnosis, suggesting a paraneoplastic mechanism.[4,5] Whether these cases represent a casual association or a true GAD-ab–associated paraneoplastic neurological syndrome (PNS) is unclear.

The discovery of antibodies against neuronal cell-surface receptors and synaptic antigens in patients with encephalitis adds complexity to the study of GAD-ab–associated neurological syndromes. Patients with LE may have coexistent GAD-abs and antibodies against the γ-aminobutyric acid (GABA) b receptor, and this association seems more frequent in patients with cancer.[6] A systematic determination of neuronal cell-surface antibodies has not been done in patients with GAD-abs and suspected PNS.

In this study, we retrospectively examined a cohort of patients with clinical criteria of definite or possible PNS but without onconeural antibodies in whom GAD-abs were identified during investigations for a paraneoplastic etiology. In addition, we performed a systematic review of previously reported cases of GAD-ab–associated PNS. The aims of this study were to describe the PNS and tumor types associated with GAD-abs, the occurrence of additional neuronal cell-surface antibodies, and the neurological response to cancer treatment and immunotherapy, as well as to provide the more frequent GAD-abs clinical settings in which a tumor screening is warranted.

Methods

Patients

In February 2014, we retrospectively identified patients examined between 1995 and 2013 with definite or possible diagnosis of PNS according to the PNS Euronetwork criteria,[7] whose serum samples were sent to our laboratory for the determination of onconeural antibodies but routine immunohistochemistry on paraformaldehyde-perfused brain tissue revealed GAD-ab reactivity (a positive brain tissue serum reactivity indicates high GAD-ab levels, usually >2000 U/mL when determined by radioimmunoassay).[3] In all samples with evidence of GAD-ab reactivity, the presence of GAD-abs was subsequently confirmed by radioimmunoassay. All patients were seen by at least 1 of the authors. Basic clinical information was obtained from medical records and additional information was collected through a structured questionnaire focused on symptom presentation, type of tumor, and response to immunotherapy and tumor treatment. Neurological disability was measured by the modified Rankin Scale[8]; a change of 1 point was required to define improvement or symptom progression.

To compare the findings of our patients with those of previously reported cases, we performed a comprehensive PubMed search using the terms *GAD antibodies AND cancer* and identified all cases until January 1, 2015. Only cases published in English that included clinical information were included. Articles were also identified by searches of the authors' files.

To define the frequency of a paraneoplastic etiology among different GAD-ab–associated neurological syndromes, we searched in our database for all cases diagnosed as having GAD-ab–associated neurological syndromes without cancer during the same period as the PNS cases were diagnosed. In total, 106 patients were identified, including 39 with cerebellar ataxia, 32 with SPS, 18 with isolated epilepsy, and 17 with LE (eFigure in the Supplement).

Patients' serum and cerebrospinal fluid (CSF) samples are deposited in the collection of biological samples named *Neuroinmunología* registered in the Biobank of Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). Written informed consent for the storage and use of the samples for research purposes was obtained from all patients. The study was approved by the ethics committee of the Hospital Clinic of Barcelona, Barcelona, Spain.

Autoantibody Assays

Levels of GAD65-abs were detected by enzyme-linked immunosorbent assay (RSR Limited) using a commercial kit following the manufacturer's specifications. Because GAD65 titers in neurologic syndromes are high, serum and CSF samples were titrated to determine the optimal dilution factor. Briefly, enzyme-linked immunosorbent assay wells were seeded for 1 hour with patients' serum samples diluted 1:10 000 or CSF diluted 1:200, followed by 1-hour incubation with GAD65 biotinylated protein, and 20-minute incubation with streptavidin peroxidase. In addition, serum and CSF samples were tested for antibodies to intracellular and neuronal cell-surface antigens using brain immunohistochemistry, as previously reported.[9,10] Onconeural antibodies to Hu, Yo, Ri, CV2, amphiphysin, and Ma1/2 were determined with immunoblot assays and GAD67, gephyrin (cotransfected 1:1 with collybistin), and neuronal cell-surface antibodies were investigated using in-house cell-based assays, including leucine-rich, glioma-inactivated 1, contactin-associated protein-like 2, GluN1/2B subunits of the *N*-methyl-D-aspartate receptor, GluR1 and 2 subunits of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate, B1 and B2 subunits of the GABAbR, α1 and β3 subunits of the GABAaR, and the α1 subunit of the glycine receptor (GlyR), as reported.[11] All DNA sequences were purchased from Origene except from those of GlyR, gephyrin, and collybistin (a gift of R. J. Harvey, PhD, Department of Pharmacology, University College London School of Pharmacy, London, England).

To demonstrate the expression of GAD65 and GAD67 in the tumor, paraffin sections were deparaffinized and the antigen retrieved, as reported.[4] After inhibition of endogenous peroxidase with 0.3% hydrogen peroxide in phosphate-buffered saline for 15 minutes, sections were incubated with GAD65 (Hybrioma Bank) or GAD67 (Abcam) monoclonal antibodies (diluted 1:1000) overnight at 4°C, and developed with the avidin-biotin peroxidase technique (Vector Laboratories).

Table 1. Clinical and Immunological Features of Patients With and Without PNS With GAD Antibodies

Characteristic	PNS (n = 15)	Non-PNS (n = 106)	*P* Value
Age, mean (range), y	60 (29-80)	48 (5-79)	.03
Female, No. (%)	6 (40)	88/102 (87)	<.001
Diabetes mellitus at onset, No./total No. (%)	2/13 (15)	31/74 (42)	.12
Other organ-specific autoimmune disorder, No./total No. (%)	4/13 (31)	43/75 (57)	.13
Clinical syndrome			
Encephalitis (LE, n = 5)[a]	6 (26)[b]	17	<.001
Cerebellar ataxia (PCD, n = 1)[a]	4 (9)[b]	39	
Stiff-person syndrome	2 (6)[b]	32	
Opsoclonus-myoclonus syndrome[a]	1 (100)[b]	0	
PEM[a]	1 (100)[b]	0	
Other[c]	1 (100)[b]	0	
Isolated epilepsy	0 (0)[b]	18	
GAD65-ab titer, median (IQR)			
Serum, $\times 10^5$ U/mL	10.5 (1.2-31.9)	5.9 (2.9-13.2)	.79
CSF, $\times 10^3$ U/mL	3.5 (1.2-57.1)	7.5 (1.7-17.2)	.86
GAD67-ab, No./total No. (%)			
Serum	8/13 (62)	93/106 (88)	.03
CSF	4/7 (57)	61/61 (100)	<.001
Neuronal cell-surface antibodies, No./total No. (%)	8/15 (53)[d]	12/106 (11)[e]	<.001

Abbreviations: CSF, cerebrospinal fluid; GAD, glutamic acid decarboxylase; IQR, interquartile range; LE, limbic encephalitis; PCD, paraneoplastic cerebellar degeneration; PEM, paraneoplastic encephalomyelitis; PNS, paraneoplastic neurological syndromes.

[a] Classic PNS.

[b] Percentage of patients in whom the syndrome was paraneoplastic.

[c] Axial rigidity, vertigo, and dysautonomia (see the Results section).

[d] γ-Aminobutyric acid bR antibody (n = 3), γ-aminobutyric acid aR antibody (n = 2), glycine receptor antibody (n = 1), and unknown antigen (n = 2).

[e] γ-Aminobutyric acid aR antibody (n = 6) and glycine receptor antibody (n = 6).

Statistical Analysis

The Fisher exact test was used to assess proportions when the expected frequencies were small (<5). For multivariate analysis of the probability of paraneoplastic origin in the present series, a logistic regression, including factors with a *P* value of .10 or less in the univariate analysis, was used. $P \leq .05$ was considered statistically significant. The software used was Stata version 13.1 (StataCorp).

Results

Fifteen patients with PNS and GAD-abs were identified. None of them had onconeural antibodies (**Table 1**). Eight of them fulfilled the criteria of definite PNS and 7 of possible PNS. The mean age was 60 years (range, 29-80 years) and 6 (40%) were women. The most frequent clinical syndromes included encephalitis (6 patients; 5 of them had typical LE[7]) and cerebellar ataxia (4 patients; 1 of them had been previously reported).[5] One patient fulfilled criteria of paraneoplastic cerebellar degeneration,[7] whereas the remaining 3 had a slowly progressive course of the disease more suggestive of degenerative ataxia. Two additional patients developed SPS, 1 opsoclonus-myoclonus syndrome, 1 paraneoplastic encephalomyelitis (previously reported[4]), and 1 a syndrome that included vertigo, ataxia, axial rigidity, and dysautonomia (eTable 1 in the Supplement).

Six patients had lung cancer (4 of them small-cell lung cancer [SCLC]), 4 had neuroendocrine tumors (2 pancreas and 2 thymic carcinoids), 2 had thymoma, 2 had breast cancer, and 1 had non-Hodgkin lymphoma. The neurological syndrome antedated the diagnosis of the cancer in 10 patients and led to the diagnosis of a tumor relapse after 10 years of remission in another patient. The median delay between the diagnosis of the neurological syndrome and cancer was 2.7 months (interquartile range, 1.2-4.5 months).

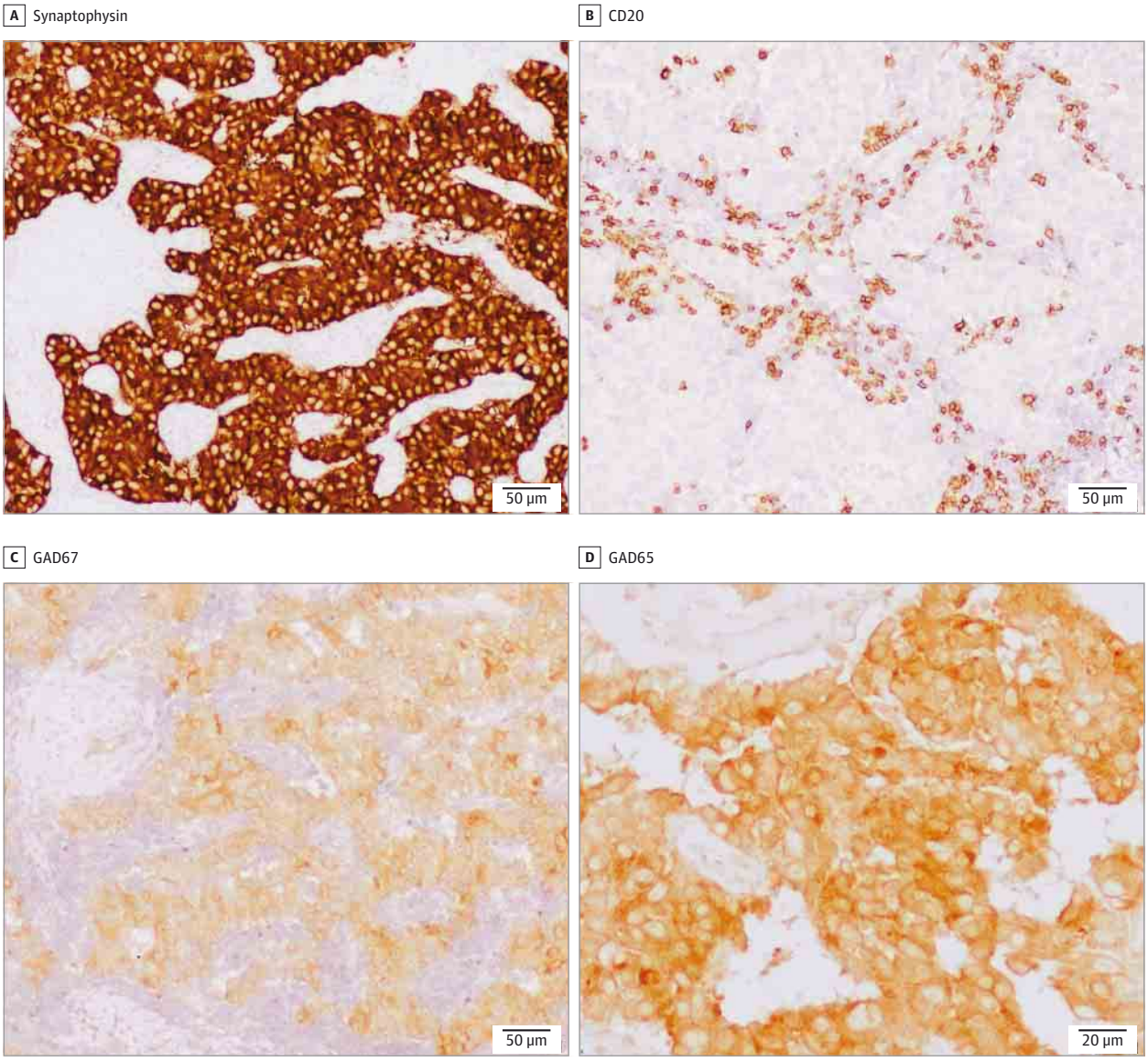
All patients received immunotherapy: 11 high-dose corticosteroids, associated with intravenous immunoglobulins in 6; 2 with isolated intravenous immunoglobulins; 1 with intravenous immunoglobulins combined with rituximab; and 1 with intravenous immunoglobulins followed by cyclophosphamide. In addition, 10 patients (71%) had oncological treatment (surgery, chemotherapy, and radiotherapy alone or combined). Clinical follow-up was available for all 15 patients: 8 (53%) had clinically progressed (5 had died owing to PNS); 4 were stable; and 3 had neurological improvement (all 3 with neoplasms of the thymus: 2 thymomas and 1 thymic carcinoid) (eTable 1 in the Supplement).

Immunological Studies

Serum and CSF GAD65-ab levels were similar in patients with or without PNS. The frequency of GAD67-abs was significantly lower in patients with PNS, particularly in CSF (Table 1). Gephyrin antibodies were not detected. Neuronal cell-surface antibodies were detected in 8 of 15 patients (53%). The antibodies, type of associated syndrome, and tumor were as follows: 3 patients with GABAbR antibodies (2 LE with SCLC and 1 paraneoplastic cerebellar degeneration with thymic carcinoma), 2 patients with GABAaR antibodies (2 LE with SCLC and thymoma), 1 patient with GlyR antibodies (cerebellar ataxia and thymic carcinoma), and 2 patients with antibodies against unknown neuronal cell-surface antigens (2 epidermoid lung cancer with opsoclonus-myoclonus syndrome and ataxia and myoclonus).

Tumor expression of GAD was assessed in 3 patients, 2 of them were previously reported.[4,5] The 3 cases had a neuroendocrine tumor (2 pancreatic and 1 thymic carcinoma) that were

Figure 1. Glutamic Acid Decarboxylase (GAD) Reactivity in a Pancreatic Tumor Sample



Immunohistochemistry (of patient 9 in eTable 1 in the Supplement) revealed the tumoral expression of synaptophysin (A) and both isoforms of GAD (C and D).

Contiguous section of the tumor immunostained with a monoclonal antibody against CD20 (B) shows infiltrates of B lymphocytes.

found to express GAD65. Only one tumor was tested for expression of GAD67 and it was found to be positive (**Figure 1**).

Comparison With Patients With GAD-abs Without Cancer

Compared with the 106 patients with nonparaneoplastic GAD-ab-associated disorders, patients with PNS were older (median age, 60 years vs 48 years; $P = .03$), were more frequently male (60% vs 13%; $P < .001$), had more often coexistent neuronal cell-surface autoantibodies (53% vs 11%; $P < .001$), and presented a different clinical profile (more classic PNS than SPS or cerebellar ataxia) (Table 1). Taking into account the clinical presentation, patients presenting with a syndrome different from SPS, cerebellar ataxia, or isolated epilepsy had a 10-fold increased risk for being paraneoplastic (odds ratio, 10.5; 95% CI, 3.2-34.5). Similarly, the detection of neuronal cell-surface antibodies carried a 7-fold increased risk for the presence of an underlying tumor (odds ratio, 6.8; 95% CI, 1.1-40.5). In a multivariate analysis that included age, sex, and the presence of neuronal cell-surface antibodies, the clinical presentation remained the most robust predictor of a paraneoplastic origin (odds ratio, 33.2; 95% CI, 5.0-220.2).

Comparison With Previously Reported GAD-ab–Associated PNS

A literature search identified 23 patients with PNS and isolated GAD-abs. Three patients[12-14] were excluded from analysis because the period between the development of PNS and tumor diagnosis was unknown or longer than 2 years.[7] An-

Table 2. Clinical Features and Tumor Associations in Patients With PNS With GAD-abs From the Present Study and Previously Reported Cases[16-34]

Feature	No. (%)		*P* Value
	Present Series (n = 15)	Literature Review (n = 19)	
Definite PNS	8	4	.05
Age, mean (range), y	60 (29-80)	56 (31-85)	.43
Sex			
Male	9	9	.46
Female	6	10	
Autoimmune diseases[a]	6	5	.39
PNS first, mo	11	17	.18
Median delay to tumor diagnosis (IQR), mo	2.7 (1.2-4.5)	1 (1-3.9)	
Clinical syndrome			
SPS (SLS)	2 (0)	14 (5)	.001
Encephalitis (LE)	6 (5)	0	
OMS	1	2	
CA (PCD)	4 (1)	1	
Other	2 (PEM and brainstem)	2 (PEM and PERM)	
Tumors			
Lung (SCLC)	6 (4)	4 (3) (Mesothelioma, n = 1)	.73
Thymoma (malignant)	4 (2)	6 (1)	
Breast	2	4	
Hematological	1 (NHL)	3 (MM, NHL, and HD)	
Other	2 (Pancreas)	2 (Kidney and cavum)	
Clinical outcome			
Improved	4	15	.005
Stable	3	0	
Worse (death)	8 (5)	4 (4)	

Abbreviations: CA, cerebellar ataxia; GAD-abs, glutamic acid decarboxylase antibodies; HD, Hodgkin disease; IQR, interquartile range; LE, limbic encephalitis; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; OMS, opsoclonus-myoclonus syndrome; PCD, paraneoplastic cerebellar degeneration; PEM, paraneoplastic encephalomyelitis; PERM, progressive encephalomyelitis, rigidity, and myoclonus; PNS, paraneoplastic neurological syndrome; SCLC, small-cell lung cancer; SLS, stiff-limb syndrome; SPS, stiff-person syndrome.

[a] Type 1 diabetes mellitus, thyroiditis, or myasthenia.

other patient was excluded because the neurological syndrome (SPS) occurred after autologous bone marrow transplantation for multiple myeloma, raising the possibility of an abnormal immune reconstitution rather than PNS as the cause of SPS.[15] The clinical information of the remaining 19 patients is summarized in eTable 2 in the Supplement.[16-34]

Among these 19 patients, 53% were female. Stiff-person syndrome was the most common neurological syndrome (74%), with a remarkable frequency of focal forms (36%). By contrast, none of the patients developed LE, which was the most prevalent syndrome in the present series. The distribution of associated tumors was also different. Thymic tumors were the most frequent neoplasm (6 patients), followed by lung cancer (4 patients) and breast cancer (4 patients). Compared with the present series, the previously reported cases appeared to have better outcomes (79% vs 27%; $P = .005$; **Table 2**).

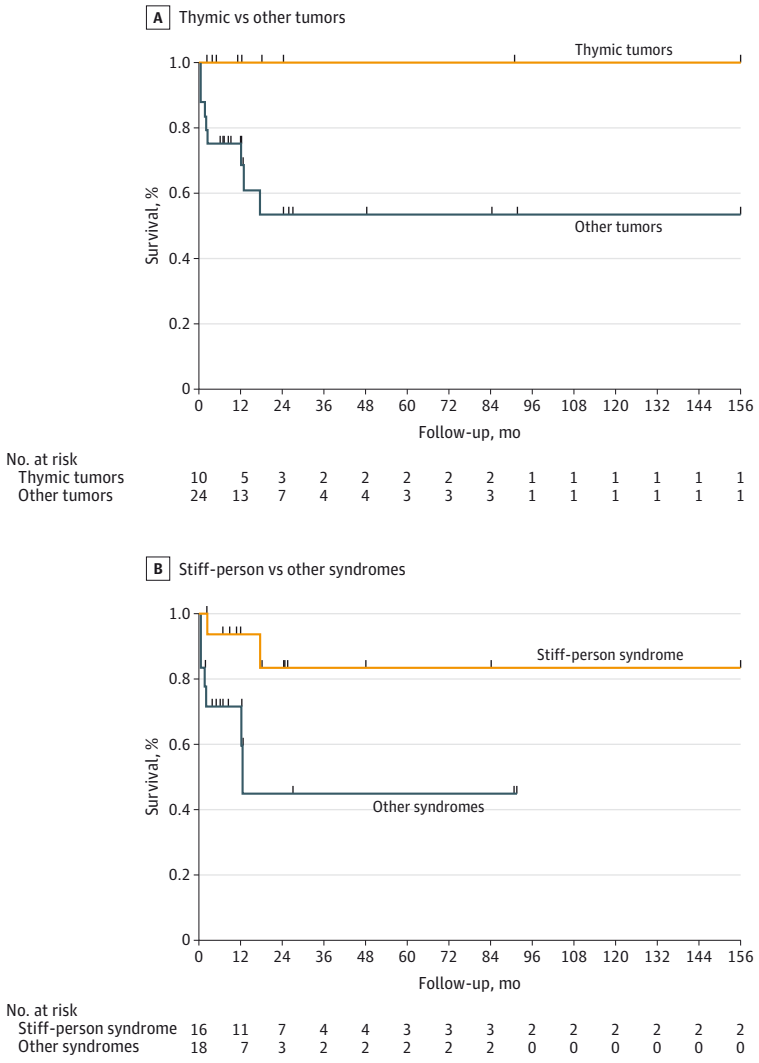
When considering together the 15 current PNS cases and the 19 previously reported, the probability of clinical improvement was greater in patients with thymic tumors, either benign or malignant (100% vs 38% other tumors; $P = .001$) and in those with SPS (75% vs 39% other syndromes; $P = .03$). Survival curves are shown in **Figure 2**.

Discussion

In some neurological syndromes with antibodies different from those strongly associated with cancer (onconeural antibodies), the immune response can be occasionally triggered by an underlying tumor and the syndrome is considered paraneoplastic. An example is LE, which, when it is associated with GABAbR-abs, may be idiopathic (autoimmune) or caused by an immune response against a tumor, usually SCLC, that expresses GABAbR.[6,11] Patients with SPS, cerebellar ataxia, or other neurological syndromes that typically associate with GAD-abs rarely have cancer and therefore an aggressive or repeated tumor search is not indicated. However, our study showed that when GAD-abs occur in patients with LE or other classic PNS (paraneoplastic cerebellar degeneration, opsoclonus-myoclonus syndrome, or paraneoplastic encephalomyelitis)[7] the risk for cancer is 10-fold higher than that in patients with SPS or cerebellar ataxia and the workup for a tumor is mandatory. The role of the tumor as a trigger of the immune response is supported by the demonstration of GAD65 in the tumors of these patients.[4,5] The higher frequency of classic PNS in our series compared with the predominance of SPS in previously published cases probably reflects the fact that our laboratory receives samples from patients with a wide spectrum of neurological syndromes, not only from those typically related to GAD-abs.

A second important observation of our study was that the probability of an underlying cancer was 7 times higher in patients with GAD-abs and coexisting antibodies against neuronal cell-surface antigens. Therefore, the determination of these antibodies in patients with GAD-abs is indicated particularly in cases of LE or cerebellar ataxia. The neuronal cell-surface antibodies predominantly identified were against GABA re-

Figure 2. Kaplan-Meyer Survival Curves of 34 Patients With Paraneoplastic Glutamic Acid Decarboxylase Syndromes by Tumor and Neurological Syndrome



A, Survival curves for 10 patients with thymic tumors compared with 24 with other tumor types. B, Survival curves for 16 patients with stiff-person syndrome and 18 with other neurological syndromes.

ceptors. γ-Aminobutyric acid bR antibodies usually occur in patients with LE and 58% of patients with these antibodies associate with cancer, mainly SCLC.[35,36] γ-Aminobutyric acid aR and GlyR antibodies have been reported in a few patients with thymoma.[37,38] However, it is important to keep in mind that with the exception of GABAbR-abs that are often detected in GAD-ab patients with cancer,[6] the antibodies against GABAaR or GlyR are more frequently found in nonparaneoplastic patients with GAD-abs.[39]

The analysis of our series and the cases previously reported identified 3 subgroups of patients in whom GAD-ab-associated syndromes can be paraneoplastic. The first group included patients with classic PNS or neurological syndromes not usually associated with GAD-abs (38%; 13 of 34 patients). These patients had additional neuronal cell-surface antibodies (46%) and lung cancer was the most frequent tumor (46%), whereas thymomas were rare (15%); only 42% of patients in this group improved with treatment. Limbic encephalitis was the most frequent PNS. It is usually considered that LE associated with GAD-abs often occurs in young women with a predominant or isolated epileptic syndrome and it is not paraneoplastic.[1] However, our study indicated that patients with LE and GAD-abs may have a tumor, usually an SCLC, and that this possibility is higher if LE occurs in older men.

The second group included patients with SPS and represented 47% (16 of 34) of all cases. Unlike patients of the first group, thymomas and breast cancer accounted for 53% of the tumors and 75% responded to therapy. Although patients with classic PNS and GAD-abs are more likely to be men, this is not the case in the subgroup of paraneoplastic SPS, where 69% of patients were women. The association of SPS with thymoma probably reflects the propensity of this tumor to induce a variety of autoimmune disorders and circulating autoantibodies.[40,41] There is no evidence that thymoma cells express GAD65 or GAD67; therefore, the possible pathogenic mechanisms remain unclear. Patients with SPS and breast cancer usually harbor amphiphysin antibodies rather than GAD-abs.[42,43] Some of the patients with SPS reported with GAD-abs and breast can-

cer also had type 1 diabetes mellitus and other organ-specific autoimmunities commonly seen in idiopathic SPS, therefore, the diagnosis of the breast cancer, a very frequent tumor, could be a coincidence. On the other hand, GAD65 is expressed in breast cancer cells and the GABAbR pathway is implicated in breast cancer cell invasion and migration.[44] This observation supports the possibility that GAD may act as a tumor antigen and thus trigger, in some patients, an immune response leading to the development of SPS.

The third group included patients with cerebellar ataxia or progressive encephalomyelitis with rigidity and myoclonus that is usually associated with GAD-abs without cancer (15%; 5 of 34 patients). The most frequent tumors were lung and breast cancer, and 2 of the 5 patients improved. These patients, as those with classic PNS, were more frequently men (80%).

A previous study on patients who underwent extensive paraneoplastic screening, including also GAD autoimmunity, identified 62 patients with GAD-abs, none of them with cancer.[45] Our experience also indicated that paraneoplastic GAD autoimmunity is infrequent[2,3] but this possibility cannot be overlooked.

Conclusions

Patients with high levels of GAD-abs (in our setting >2000 U/mL by radioimmunoassay) and classic PNS or neurological syndromes not typically associated with GAD-abs should be screened for an underlying cancer. Considering the tumors identified in the current series and previous reported cases, the tumor workup should include mammogram and chest computed tomography or computed tomography–positron emission tomographic scan, depending on the clinical setting. The cancer risk increases with age, male sex, and presence of concomitant antibodies against neuronal cell-surface antigens.

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Acquisition, analysis, or interpretation of data: All authors.
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Critical revision of the manuscript for important intellectual content: All authors.
Statistical analysis: Ariño.
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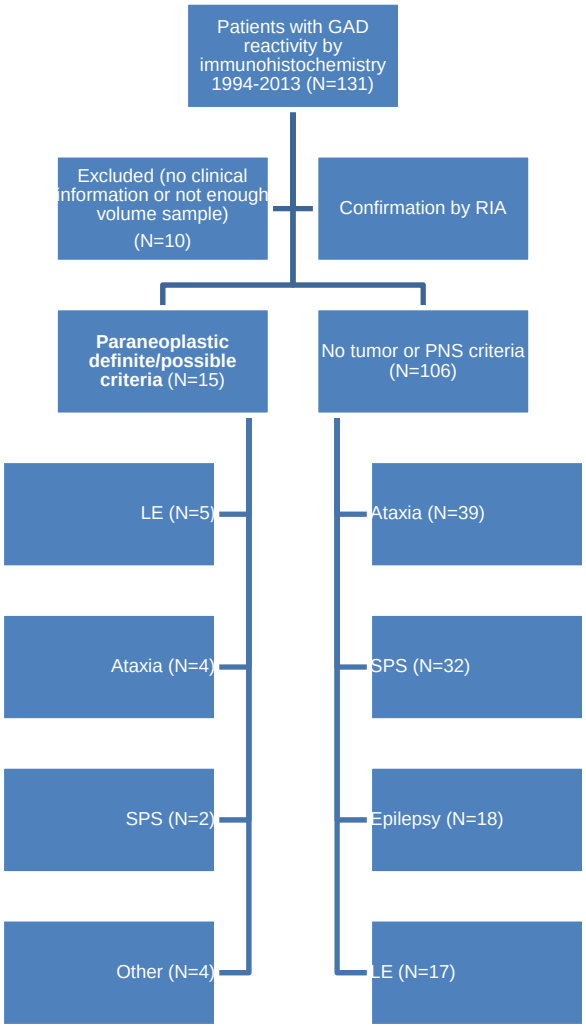
Supplementary Online Content

Ariño H, Höftberger R, Gresa-Arribas N, et al. Paraneoplastic neurological syndromes and glutamic acid decarboxylase antibodies. *JAMA Neurol*. Published online June 22, 2015. doi:10.1001/jamaneurol.2015.0749.

This supplementary material has been provided by the authors to give readers additional information about their work.

eFigure. Flowchart of Patients



RIA: radioimmunoassay; PNS: paraneoplastic syndrome; LE: limbic encephalitis; SPS: stiff-person syndrome.

eTable 1. Patients of Our Series With Paraneoplastic Neurological Syndromes and GAD Antibodies

Patient	Age/Sex	Syndrome	PNS level	First diagnosis (delay in months)	Tumor	Treatment	Outcome (follow-up in months)	Comments
1	80/M	LE	Definite	PNS (3)	SCLC (relapse)	Steroids, chemotherapy	Worse (6)	Thyroiditis
2	79/M	CA	Possible	PNS (2)	NSCLC	IVIg, steroids	Worse (7)	
3	54/M	CA	Possible	PNS (6)	Thymic neuroendocrine carcinoma[a]	Surgery, chemotherapy, RT, IVIg, steroids, rituximab	Improved (91)	Hypothyroidism and diabetes years later
4	57/F	PCD	Definite	PNS (1)	Thymic neuroendocrine carcinoma	Steroids, IFN alpha-2b	Improved (4)	Hypothyroidism
5	67/M	PEM	Definite	PNS (3)	Pancreatic neuroendocrine carcinoma[a]	Surgery, IVIg, diazepam, baclofen, gabapentine	Death (12)	
6	61/F	CA	Possible	Tumor (15)	Breast	Surgery, chemotherapy, RT, IVIg, PLEX, cyclophosphamide	Stable (91), relapse 3 years later, response to immunotherapy	Myasthenia
7	73/F	LE	Definite	PNS (2)	NHL (Burkitt)	Steroids, IVIg	Death (3)	Dysautonomia
8	70/M	LE	Definite	PNS (1)	SCLC	Steroids, IVIg,	Death (2)	
9	78/F	Brainstem[b]	Possible	PNS (5)	Pancreatic neuroendocrine carcinoma[a]	Surgery, chemotherapy, IVIg	Stable (27)	
10	66/M	LE	Definite	PNS (1)	SCLC	Steroids, IVIg	Death (13)	
11	37/M	LE	Definite	PNS (3)	Thymoma	Surgery, steroids	Improved (4)	Later developed

								SPS
12	72/M	OMS	Definite	Tumor (1)	NSCLC	Surgery, chemotherapy, IVIg, steroids	Death (1)	
13	47/M	SPS	Possible	Tumor (27)[c]	SCLC	Chemotherapy, steroids	Stable (9)	Diabetes
14	40/F	SPS	Possible	PNS (24)	Breast	Surgery, chemotherapy, IVIg, steroids, diazepam	Worse (84)	Thyroiditis, CA 5 years later
15	29/F	Encephalitis[d]	Possible	Tumor (19)	Thymoma	Surgery, steroids, PLEX	Improved (2)	Myasthenia

PNS: paraneoplastic neurological syndrome; LE: limbic encephalitis; PCD: paraneoplastic cerebellar degeneration; PEM: paraneoplastic encephalomyelitis, SCLC: small-cell lung cancer; NSCLC: non-small cell lung cancer; HNL: non-Hodgkin lymphoma; SPS: stiff-person syndrome; RT radiotherapy; PLEX: plasma exchange: IVIg: intravenous immunoglobulins; OMS: opsoclonus-myoclonus syndrome

[a] Tumor cells expressed GAD65; [b] Vertigo, ataxia, axial rigidity, and dysautonomia; [c] Tumor active at the time of PNS diagnosis; [d] Sleepiness, aphasia, left faciobrachial paresis and dystonic movements in both legs with a MRI showing multifocal cortico-subcortical hyperintensities in FLAIR sequences that improved in a second MRI.

eTable 2. Literature Survey of Patients With Paraneoplastic Neurological Syndromes and GAD Antibodies[1–19]

Author (year)	Age/ sex	Syndrome	PNS level	First diagnosis (delay in months)	Tumor	Treatment	Outcome (follow-up in months)	Comments
Ferrari (1990)	31/M	SPS	Possible[a]	PNS (1)	Hodgkin (relapse)	Chemotherapy, diazepam, baclofen	Improved (12)	
Silverman (1999)	68/F	SPS (SLS)	Possible	PNS (1)	Breast	Steroids, clonazepam	Death (18)	Grave's disease. Autopsy: no inflammation
Hagiwara (2001)	40/F	SPS	Possible	PNS (1)	Thymoma	Surgery, RT steroids, immunoadsorption	Improved (?)	
Sinnreich (2001)	85/F	SPS	Possible	PNS (6)	Breast	Surgery, PLEX, IVIg, steroids	Improved (7)	Hashimoto ; uveitis
Schiff (2003)	47/F	SPS (SLS)	Possible	Tumor relapse (9)	Multiple myeloma	Diazepam	Improved (24)	Hypothyroidism, epilepsy
Thomas (2005)	45/M	SPS	Possible[a]	PNS (11)	Thymoma	Surgery, baclofen, IVIg, clonazepam	Improved (18)	
Tanaka H (2005)	57/F	SPS	Possible[a]	PNS (4)	Thymoma	Diazepam, surgery baclofen	Improved (24)	SPS relapse, responded to IVIg
Iwata T (2006)	79/F	SPS (SLS)	Possible[a]	PNS (3)	Thymoma	Surgery, IVIg	Improved (12)	A relapse treated with PE
McHugh (2007)	53/M	SPS (SLS)	Possible	PNS (11)	Kindey	IVIg , baclofen, diazepam	Improved (48)	Hypothyrodism and diabetes
Agarwal (2010)	55/F	SPS (SLS)	Possible[a]	PNS (2)	Breast	Chemotherapy, steroids, baclofen,	Improved (12)	

						diazepam		
Rakocevic (2012)	44/F	SPS	Possible [c]	Tumor (48)[b]	Cutaneous T cell lymphoma	Baclofen, IVIg, diazepam, steroids, cyclophosphamide, tacrolimus,	Improved (156)	rituximab and alemtuzumab for relapses
Aghajanzadeh (2013)	32/M	SPS	Definite [a]	PNS (5)	Thymic carcinoma	Surgery	Improved (11)	Baclofen and diazepam not effective
Koca (2014)	58/F	SPS	Possible [a]	Tumor (?)[d]	Mesothelioma	Diazepam, baclofen, steroids	Death (3)	SPS did not improve
Kobayashi (2014)	68/M	SPS	Possible [a]	PNS (1)	Thymoma	Surgery, steroids	Improved (156)	Relapse 13 years later at the time of tumor recurrence
Spitz (2004)	73/M	PERM	Possible [a]	PNS (0.5)	SCLC	Chemotherapy, IVIg, diazepam	Death (1)	
Venker (2011)	52/M	CA and dystonia	Possible [a]	PNS (11)	Breast (relapse)	Chemotherapy, immunoabsortion	Improved (13)	
Carra-Dalliere (2012)	59/M	PEM	Definite	PNS (1)	SCLC	Chemotherapy, RT, IVIg	Improved (12)	
Laroumagne (2014)	65/M	OMS	Definite	PNS (0.5)	SCLC	Chemotherapy, steroids	Death (1)	
Lamotte (2014)	61/M	OMS	Definite	PNS (0.5)	Pyriform sinus	Chemotherapy, RT, IVIg	Improved (6)	

PNS; paraneoplastic neurological syndrome; SLS, stiff-limb syndrome; SPS: Stiff-person syndrome; RT: radiotherapy; PLEX: plasma exchange; IVIg: intravenous immunoglobulins; PERM: progressive encephalomyelitis with rigidity and myoclonus; PEM: paraneoplastic encephalomyelitis; SCLC: small-cell lung cancer; OMS: opsoclonus-myoclonus syndrome

[a] Amphiphysin antibodies not tested; [b] tumor active at the onset of the PNS; [c] PNS improved each time tumor was put into remission; [d] tumor active at the time of SPS diagnosis.

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Paper IV

Clinical and Immunologic Investigations in Patients With Stiff-Person Spectrum Disorder.

Martinez-Hernandez E, Ariño H, McKeon A, Iizuka T, Titulaer MJ, Simabukuro MM, Lancaster E, Petit-Pedrol M, Planagumà J, Blanco Y, Harvey RJ, Saiz A, Graus F, Dalmau J. JAMA Neurol. 2016 Jun 1;73(6):714-20.

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Contribution:

We examined the largest cohort of patients with stiff-person spectrum disorders. The relative frequency of several antibodies, syndrome specificity, and prognostic implications were investigated. Our data showed that the immunologic characterization may be helpful to predict the outcome, with independence of the clinical classification.

Original Investigation

Clinical and Immunologic Investigations in Patients With Stiff-Person Spectrum Disorder

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IMPORTANCE Symptoms of stiff-person syndrome (SPS), stiff-limb syndrome (SLS), or progressive encephalomyelitis with rigidity, myoclonus, or other symptoms (SPS-plus) can occur with several autoantibodies, but the relative frequency of each antibody, syndrome specificity, and prognostic implications are unclear.

OBJECTIVE To report the clinical and immunologic findings of a large cohort of patients with stiff-person spectrum disorder (SPSD), including SPS, SLS, and SPS-plus.

DESIGN, SETTING, AND PATIENTS This study retrospectively examined a case series (January 1, 1998, through December 31, 2014) of immunologic investigations performed in a neuroimmunology referral center. The study included 121 patients with clinical features of SPSD. Data analysis was performed from July 1, 2015, through November 1, 2015.

MAIN OUTCOMES AND MEASURES Analysis of clinical-immunologic associations, including autoantibodies to 8 proteins expressed in inhibitory synapses.

RESULTS The median age of the patients was 51 years (interquartile range, 40-61 years), and 75 (62.0%) were female. Fifty (41.3%) had SPS, 37 (30.6%) had SPS-plus, 24 (19.8%) had SLS, and 10 (8.3%) had SPS or SLS overlapping with ataxia, epilepsy, or encephalitis. Fifty-two patients (43.0%) had glutamic acid decarboxylase (GAD65) antibodies (2 with γ-aminobutyric acid–A [GABA-A] receptor antibodies), 24 (19.8%) had α_1-subunit of the glycine receptor (GlyR) antibodies (2 with GAD65 antibodies), 5 (4.1%) had other antibodies, and 40 (33.1%) tested negative for antibodies. None had gephyrin or glycine transporter antibodies. Among the main immunologic groups (GAD65 antibodies, GlyR antibodies, and antibody negative), those with GAD65 antibodies were more likely to be female (45 [86.5%] of 52, 8 [36.4%] of 22, and 18 [45.0%] of 40, respectively; $P < .001$), have systemic autoimmunity (34 [65.4%] of 52, 7 [31.8%] of 22, and 13 [32.5%] of 40, respectively; $P = .004$), and have longer delays in being tested for antibodies (median, 3 vs 0.5 and 1 year; $P < .001$). Patients with GAD65 antibodies were more likely to develop SPS (27 [51.9%] of 52) or overlapping syndromes (8 [15.4%] of 52) than patients with GlyR antibodies (5 [22.7%] and 0 [0%] of 22, respectively), who more often developed SPS-plus (12 [54.5%] of 22 vs 7 [13.5%] in those with GAD65 antibodies); antibody-negative patients had an intermediate syndrome distribution. In multivariable analysis, symptom severity ($P = .001$) and immunologic group ($P = .01$) were independently associated with outcome. Compared with patients with GlyR antibodies, those with GAD65 antibodies (odds ratio, 11.1, 95% CI, 2.3-53.7; $P = .003$) had worse outcome. Patients without antibodies had similar outcome than patients with GlyR antibodies (odds ratio, 4.2, 95% CI, 0.9-20.0; $P = .07$).

CONCLUSIONS AND RELEVANCE In SPSD, symptom severity and presence and type of antibodies are predictors of outcome.

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Stiff-person syndrome (SPS) is a disorder characterized by fluctuating muscle rigidity and painful spasms that occur spontaneously or are triggered by diverse stimuli.[1,2] Partial or segmental forms of the disorder, such as stiff-limb syndrome (SLS) and the more severe disease called progressive encephalomyelitis with rigidity and myoclonus (PERM), are usually considered within the spectrum of SPS,[3-6] but there is an increasing recognition of atypical and overlapping syndromes. For all these disorders, which we collectively termed *stiff-person spectrum disorder* (SPSD), there is evidence of underlying immune mechanisms that target proteins mainly expressed by the inhibitory synapses. Six autoantigens have been identified, including glutamic acid decarboxylase (GAD65),[7,8] the α_1-subunit of the glycine receptor (GlyR),[9,10] amphiphysin,[11] gephyrin,[12] dipeptidyl peptidase-like protein 6 (DPPX),[13,14] and the γ-aminobutyric acid-A (GABA-A) receptor (GABAaR).[15] Some of these immune responses have been suggested to be associated with distinct variants of SPSD,[16] but the degree of syndrome specificity and implications for treatment and prognosis are unclear. Because some autoantigens were recently discovered and SPS is a rare disease, most studies have focused on a limited number of autoantibodies (GAD65 or GlyR) and well-defined syndromes (SPS or PERM) without examining the entire spectrum of clinical-immunologic associations and the implications of being antibody negative. To address these issues, we investigated the clinical features of 121 patients with SPSD, determined the presence of autoantibodies to 8 potential targets of the inhibitory synapse, and compared the syndromes among the most frequent immunophenotypes. In addition, we provide the treatment, outcome, and prognostic factors of 75 patients for whom long-term follow-up information was available.

Key Points

Question Do autoantibodies have prognostic implications in stiff-person spectrum disorder (SPSD)?

Findings In this retrospective case series that included 121 patients, those with glutamic acid decarboxylase antibodies had worse outcome than patients with glycine receptor antibodies or patients who tested negative for antibodies. In contrast, the type of syndrome did not predict outcome.

Meaning In SPSD, the presence and type of autoantibodies are predictors of outcome.

Methods

Study Design and Participants

We retrospectively reviewed the clinical information of patients with SPSD seen by us (57 cases) or whose serum or cerebrospinal fluid (CSF) samples were referred to our laboratory for antibody testing from January 1, 1998, through December 31, 2014. Data analysis was performed from July 1, 2015, through November 1, 2015. Stiff-person spectrum disorder was clinically defined by the presence of symptoms of axial stiffness and muscle spasms not restricted to the classic presentation of SPS but also including forms with partial or distal limb distribution or symptoms of encephalomyelitis. Clinical information was obtained by us or the referring physicians with a structured questionnaire.

One hundred forty-six patients were initially identified as having possible SPSD. Of these, 20 were excluded after other disorders were identified (eMaterial in Supplement) and another 5 because of suboptimal information. Overall, 121 patients had the final diagnosis of SPSD, comprising 4 groups: (1) classic SPS: rigidity in axial trunk, sometimes involving proximal limbs, in association with muscle spasms, resulting in abnormal axial posture[1]; (2) SLS: affecting 1 or more limbs with distal rigidity and abnormal posturing of hands or feet[5]; (3) SPS-plus, including patients with all or partial elements of PERM: brainstem dysfunction, myoclonus, upper or lower motor neuron symptoms, sensory deficits, sphincter or autonomic dysfunction, seizures, and cognitive changes[6]; and (4) overlapping syndromes, including patients with SPS or SLS in association with cerebellar ataxia, epilepsy, or limbic encephalitis.[17-20]

Treatments were classified as (1) symptomatic (eg, GABAergic drugs), (2) first-line immunotherapies (intravenous corticosteroids, intravenous immunoglobulin, or plasma exchange alone or combined), (3) second-line immunotherapies (rituximab, cyclophosphamide), and (4) long-term oral immunotherapy (prednisone, azathioprine, mycophenolate mofetil, cyclosporine).

The delay to diagnosis was calculated as the difference between the patient's age at the time of autoantibody testing and the age at symptom onset. Neurologic disability was measured using the modified Rankin Scale (mRS).[21] The degree of improvement was calculated as the difference between the maximum mRS score during the disease and the score at the last follow-up. Improvement of at least 1 point with an mRS score of 0 to 2 at the last follow-up was considered a good outcome; a score of 3 or higher or no change in the mRS score was considered a bad outcome. Written informed consent for studies was obtained from patients or their families. The study was approved by the institutional review boards of the Hospital of the University of Pennsylvania and Hospital Clinic, University of Barcelona.

Antibody Assays

Paired serum and CSF samples were available from 65 patients, only serum from 50, and only CSF from 6. Antibody studies were performed using previously reported techniques, which are described in the eMaterial in the Supplement. Serum or CSF samples of 245 patients were used as controls, including samples from 30 healthy individuals, 20 patients with neurodegenerative diseases, and 195 patients with immune-mediated central nervous system disorders (eMaterial in Supplement).

Statistical Analysis

Demographic information and symptoms were analyzed using the Fisher exact test, Fisher-Freeman-Halton test (an extension for rxc contingency tables), or Mann-Whitney test,

as appropriate. Because of a skewed distribution, log transformation was used for duration of follow-up. The maximum mRS score was dichotomized as low (range, 0-3) or high (range, 4-6). Factors that influenced outcome were assessed by univariable binary logistic regression. Factors associated with a bad outcome ($P < .10$) were included in a multivariable binary logistic regression model and approached by backward stepwise procedure; only the variables that remained significant were considered as independent predictors. Odds ratios (95% CIs) were used to measure the effect of predictors. SPSS statistical software, version 19 (SPSS Inc), was used for the analyses.

Results

General Clinical Features

Seventy-five (62.0%) of 121 patients were women. The median age at symptom onset was 51 years (interquartile range [IQR], 40-61 years), and the median delay to diagnosis was 2 years (IQR, 0-4 years). Fifty patients (41.3%) had SPS, 37 (30.6%) had SPS-plus, 24 (19.8%) had SLS, and 10 (8.3%) had SPS or SLS overlapping with cerebellar ataxia (6 cases), epilepsy (3 cases), or limbic encephalitis (1 case). Clinical features, diagnostic tests, and outcome according to immunologic groups are listed in **Table 1** and according to the type of syndrome in eTable 1 in the Supplement.

Neurophysiologic studies were performed in 84 patients, revealing continuous motor unit activity in 52 (61.9%), with a similar proportion of positive cases across all syndromes (eTable 1 in the Supplement). Only 7 patients (5.8%) had abnormal magnetic resonance imaging findings, including high T2 signal in temporal lobes in 2 patients with overlapping syndromes (1 limbic encephalitis and 1 epilepsy) and focal or diffuse high T2 signal in 5 patients with SPS-plus (2 brain, 2 brainstem, and 1 spinal cord abnormalities). Three patients (2.5%) had cancer (2 breast, 1 colon), 1 each presenting with SLS, SPS-plus, and overlapping syndromes.

Patients with SPS-plus had higher mRS scores (more disabled) than those with SPS and SLS (median, 4; IQR, 3-5; median, 4; IQR, 2.5-4 [$P = .001$]; and median, 3; IQR, 2-4 [$P = .002$], respectively) (eTable 1 in the Supplement). In addition to first-line immunotherapy, patients with SPS-plus were more likely to receive long-term oral immunotherapy than those with other syndromes, and patients with overlapping neurologic syndromes were more likely to receive second-line immunotherapy (eTable 1 in the Supplement). Symptomatic GABAergic drugs were more frequently used in typical SPS (42 [84.0%] vs 16 [66.6%] in SLS, 21 [56.7%] in SPS-plus, and 5 [50.0%] in overlapping syndromes, $P = .006$).

Antibody Findings

Eighty-one patients (66.9%) had autoantibodies against inhibitory synaptic proteins, including 52 (43.0%) with GAD65 (2 with concurrent GABAaR antibodies), 24 (19.8%) with GlyR (2 with concurrent GAD65 antibodies), 5 (4.1%) with other antibodies (2 GABAaR, 2 amphiphysin, 1 DPPX), and 40 (33.1%) who were antibody negative. Paired serum and CSF samples were studied in 26 (50.0%) of 52 patients with GAD65 antibodies and 14 (58.3%) of 24 patients with GlyR antibodies; in 3 (11.5%) of 26 patients, GAD65 antibodies were detected only in CSF, and in 6 (42.8%) of 14 patients, GlyR antibodies were detected only in serum. None of the patients had antibodies against gephyrin, glycine transporter (GlyT) 1 or GlyT2. Three patients (2.4%) of 121, without antibodies against any of the 8 target antigens, had serum antibodies against unknown neuronal cell surface antigens determined in live neuronal cultures.

Fifteen (6.1%) of 245 controls had GlyR antibodies in serum: 4 had cerebellar ataxia, 2 had epilepsy, 4 had anti-*N*-methyl-D-aspartate receptor encephalitis, and 5 had multiple sclerosis. Five controls (2.0%) had GABAaR antibodies: 1 had cerebellar ataxia, 3 had epilepsy, and 1 had anti-*N*-methyl-D-aspartate receptor encephalitis. Paired CSF samples were available from 12 of these 20 patients, and all tested negative for GlyR and GABAaR antibodies. The titers of GlyR antibodies in serum tended to be lower in the controls than in patients with SPSD (median titer, 1/80; range, 1/40 to 1/640; vs median titer, 1/160; range, 1/40 to 1/1280; $P = .06$). The titers of GABAaR antibodies in serum of controls and patients with SPSD were similar (median titer, 1/40; range, 1/20 to 1/160; vs median titer, 1/40; range, 1/20 to 1/40; $P = .09$). None of the controls with GlyR antibodies had symptoms of SPSD; 4 of 5 controls with serum GABAaR antibodies had prominent seizures.

Clinical Comparisons of Immunologic Groups

These studies focused on the main immunologic groups (GAD65 antibodies, GlyR antibodies, and antibody negative), which comprised 114 (94.2%) of 121 patients; the 2 patients with coexisting GAD65 and GlyR antibodies were excluded. Patients with GAD65 antibodies or antibody-negative patients were more likely to develop SPS than those with GlyR antibodies, who more frequently developed SPS-plus ($P = .002$; see list of symptoms in Table 1). Patients with GAD65 antibodies were investigated for antibodies later than those with GlyR antibodies or antibody-negative patients (median delay to diagnosis, 3 years; IQR, 1-6 years; 0.5 year; IQR, 0-2 years [$P < .001$]; and 1 year; IQR, 0-5 years [$P = .02$], respectively) and had lower maximum mRS scores (lower symptom severity) compared with those of the other 2 groups (median mRS score for GAD65, 3.5; IQR, 2-4; GlyR, 4; IQR, 3.5-5 [$P = .01$]; and antibody negative, 4; IQR, 3-5 [$P = .05$], respectively). Patients with GAD65 antibodies were more likely to be female (45 [86.5%] of 52, 8 [36.4%] of 22, and 18 [45.0%] of 40, respectively; $P < .001$) and had more frequent systemic autoimmune or endocrine disorders (34 [65.4%] of 52, 7 [31.8%] of 22, and 13 [32.5%] of 40, respectively; $P = .004$). On the other hand, patients with GlyR antibodies had more frequent CSF pleocytosis than those of the other 2 groups (7 [38.9%] of 18 vs 2 [10.0%] of 20 for the GAD65 group and 4 [14.8%] of 27 for the antibody-negative group, $P < .001$). None of the antibody-negative patients had CSF oligoclonal bands (11 [47.8%] of 23, 5 [29.4%] of 17, and 0 of 19 in the GAD65 antibodies, GlyR antibodies, or antibody-negative groups, respectively; $P < .001$). No significant differences were identified in terms of age at onset of symptoms, electrophysiologic findings, immunotherapies used, and relapses among the 3 immunological groups (Table 1).

Table 1. Clinical Features of the Main Immunologic Groups[a]

Clinical Feature	GAD65 Antibodies (n = 52 [43.0%])	GlyR Antibodies (n = 22 [18.2%])[b]	Antibody Negative (n = 40 [33.1%])	*P* Value
Female sex	45 (86.5)	8 (36.4)	18 (45.0)	<.001
Age at onset, median (IQR), y	52.5 (39.0-62.0)	46.0 (39.0-60.0)	50.0 (39.0-59.0)	.74
Delay to diagnosis, median (IQR), y	3.0 (1.0-6.0)	0.5 (0-2.0)	1.0 (0-5.0)	<.001[c]
Syndrome				
SPS	27 (51.9)	5 (22.7)	17 (42.5)	.002
SLS	10 (19.2)	5 (22.7)	7 (17.5)	
SPS-plus	7 (13.5)	12 (54.5)	15 (37.5)	
Overlapping syndromes	8 (15.4)	0	1 (2.5)	
Hyperekplexia	14 (26.9)	11 (50.0)	11 (27.5)	.009
Myoclonus	6 (11.5)	9 (40.9)	9 (22.5)	.07
Brainstem	3 (5.8)	10 (45.5)	7 (17.5)	.002
Pyramidal	7 (13.5)	11 (50.0)	11 (27.5)	.004
Sphincter	1 (1.9)	7 (31.8)	5 (12.5)	.008
Sensory	0	5 (22.7)	3 (7.5)	.01
Autonomic	5 (9.6)	7 (31.8)	6 (15.0)	.06
Insomnia	2 (3.8)	6 (27.3)	7 (17.5)	.045
Systemic autoimmune disorders[d]	34 (65.4)	7 (31.8)	13 (32.5)	.004
CMUA on EMG	26/36 (72.2)	6/14 (42.9)	16/30 (53.3)	.25
CSF pleocytosis	2/20 (10.0)	7/18 (38.9)	4/27 (14.8)	<.001
CSF OCBs	11/23 (47.8)	5/17 (29.4)	0/19 (0)	<.001
Maximum mRS score, median (IQR)	3.5 (2-4)	4 (3.5-5)	4 (3-5)	.02[c]
Symptomatic treatment	46 (88.5)	10 (45.5)	24 (60.0)	<.001
Type of immunotherapy				
Nontreated	9/45 (20.0)	2/18 (11.1)	6/28 (21.4)	.17
First line only	22/45 (48.9)	4/18 (22.2)	11/28 (29.3)	
First line and long-term oral	4/45 (8.9)	7/18 (38.9)	4/28 (14.3)	
First and second line	7/45 (15.5)	5/18 (27.8)	6/28 (21.4)	
Other[e]	3/45 (6.7)	0	1/28 (3.6)	
Cases with follow-up	41 (78.8)	14 (63.6)	24 (60.0)	.13
Follow-up period, median (IQR), mo	34.0 (12.0-66.0)	11.0 (8.0-40.0)	12.0 (9.0-54.0)	.049[c]
Relapsing course	13/41 (31.7)	6/14 (42.9)	6/24 (25.0)	.77
Final mRS score, median (IQR)	3 (1-3)	1 (1-3)	2.5 (1-4)	.14
Improvement				
Treated	24/32 (75.0)	11/14 (78.6)	11/19 (57.9)	.14
Nontreated	3/9 (33.3)	0	4/5 (80.0)	
Change in mRS score, median (IQR)	1 (0-1)	3 (0.75-4)	1 (0-2)	.009[c]

Abbreviations: CMUA, continuous motor unit activity; CSF, cerebrospinal fluid; EMG, electromyography; GAD65, glutamic acid decarboxylase; GlyR, α_1-subunit of the glycine receptor; IQR, interquartile range; mRS, modified Rankin scale; OCBs, oligoclonal bands; SLS, stiff-limb syndrome; SPS, stiff-person syndrome; SPS-plus, progressive encephalomyelitis with rigidity, myoclonus, or other symptoms.

[a] Data are presented as number (percentage) of patients unless otherwise indicated.

[b] Excludes 2 patients with concurrent GAD65 and GlyR antibodies.

[c] Mann-Whitney test: delay to diagnosis for patients with GAD65 antibodies vs GlyR antibodies, $P < .001$; GAD65 antibodies vs antibody negative, $P = .02$; and GlyR antibodies vs antibody negative, $P = .052$; maximum mRS score for patients with GAD65 antibodies vs GlyR antibodies, $P = .01$; GAD65 antibodies vs antibody negative, $P = .05$; and GlyR antibodies vs antibody negative, $P = .44$; follow-up period for patients with GAD65 antibodies vs GlyR antibodies, $P = .04$; GAD65 antibodies vs antibody negative, $P = .053$; and GlyR antibodies vs antibody negative, $P = .70$; and change in mRS score for GAD65 antibodies vs GlyR antibodies, $P = .002$; GAD65 antibodies vs antibody negative, $P = .41$; and GlyR antibodies vs antibody negative, $P = .04$.

[d] Fifty-four patients (44.6%), including 18 with type 1 diabetes mellitus, 13 with thyroiditis, 2 with celiac disease, 2 with psoriasis, 2 with vitiligo, 2 with Raynaud syndrome, and 15 with thyroid, antinuclear antibody, double-stranded DNA, or gastric parietal cell antibodies.

[e] Includes 3 patients who received long-term oral immunotherapy (not preceded by first-line therapy) and 1 patient who received chemotherapy for colon cancer.

Because SPSD without antibodies has been infrequently reported in the literature,[16] we further assessed the clinical features of the 3 immunologic groups to compare only those patients with SPSD and electromyographic findings of agonist-antagonist continuous motor unit activity. This subgroup analysis revealed that the antibody-negative patients still composed one-third of the cases, and the main distinctive clinical features among immunologic groups were similar to those indicated above (eTable 2 in the Supplement).

Among the group of 10 patients with SPS or SLS, and overlapping syndromes, 8 had GAD65 antibodies (5 cerebellar ataxia and 3 epilepsy), 1 had limbic encephalitis and

Table 2. Variables Related to a Bad Outcome (mRS Scores of 3-6 or No Improvement)

Variable	Odds Ratio (95% CI)	*P* Value
Univariable Analysis		
Sex	1.56 (0.59-4.1)	.37
Age at onset	1.02 (0.99-1.05)	.18
Clinical syndrome		.85
SPS (n = 32)	...	...
SLS (n = 14)	0.58 (0.16-2.1)	.40
SPS-plus (n = 25)	0.72 (0.25-2.1)	.54
Overlapping syndromes (n = 4)	... (0-∞)	.99
Delay to diagnosis[a]	1.20 (0.96-1.48)	.11
Maximum mRS group (4-6 vs 0-3)	3.9 (1.50-10.4)	.005
Follow-up period (log_e)	0.89 (0.57-1.41)	.63
Immunotherapy		
Nontreated	...	...
Treated	1.18 (0.37-3.8)	.78
Immunologic group		.08
GlyR antibodies (n = 14)	...	...
Antibody negative (n = 22)	2.5 (0.60-10.4)	.21
GAD65 antibodies (n = 39)	4.5 (1.20-16.9)	.03
Multivariable Analysis		
Maximum mRS group	7.5 (2.3-24.5)	.001
Immunologic group		.01
GlyR antibodies	...	...
Antibody negative	4.2 (0.90-20.0)	.07
GAD65 antibodies	11.1 (2.3-53.7)	.003

Abbreviations: ellipses, data not applicable; GAD65, glutamic acid decarboxylase; GlyR, α_1-subunit of the glycine receptor; log_e, natural logarithm; mRS, modified Rankin scale; SLS, stiff-limb syndrome; SPS, stiff-person syndrome; SPS-plus, progressive encephalomyelitis with rigidity, myoclonus, or other symptom.

[a] For patients tested for autoantibodies after 5 years of symptom onset (n = 15), the delay to diagnosis was considered 6 years.

amphiphysin antibodies, and 1 had cerebellar ataxia and was antibody negative. Five patients without GAD65 and GlyR antibodies had autoantibodies against other known antigens (2 amphiphysin, 2 GABAaR, and 1 DPPX; eMaterial in Supplement).

Clinical Outcome

Clinical outcome was available for 75 patients (62.0%), with a median follow-up of 18 months (IQR, 11-60 months). Patients with GlyR antibodies had a greater degree of improvement than those with GAD65 antibodies or without antibodies (median change in mRS score for patients with GlyR antibodies, 3; IQR, 0.75-4; for patient with GAD65 antibodies, 1; IQR, 0-1 [*P* = .002]; and antibody-negative patients, 1; IQR, 0-2 [*P* = .04]) (Table 1). The eFigure in the Supplement shows patients' outcome according to the clinical syndrome and immunologic group. At the last follow-up, 40 patients (53.3%) had a bad outcome, and 35 (46.7%) had a good outcome. Nine patients (12.0%) died, 5 as a result of systemic complications (pneumonia, pulmonary embolism, sepsis, intestinal perforation, status epilepticus) and 4 of cardiorespiratory arrest; 6 patients had GAD65 antibodies, 2 were antibody negative, and 1 had GlyR antibodies. In univariable analysis, the factors significantly associated with a bad outcome were symptom severity and presence of GAD65 antibodies (**Table 2**). In multivariable analysis, symptom severity and immunologic group were independently associated with outcome (Table 2). Compared with patients with GlyR antibodies, those with GAD65 antibodies (odds ratio, 11.1, 95% CI, 2.3-53.7; *P* = .003) had worse outcome. Patients without antibodies had similar outcome than patients with GlyR antibodies (odds ratio, 4.2, 95% CI, 0.9-20.0; *P* = .07).

Discussion

This study reveals that in a cohort of 121 patients with SPSD only 50 (41.3%) had typical SPS, and the overall prognosis depended more on the underlying immune mechanism and severity of symptoms than on the type of syndrome. Among 8 potential autoantigens, GAD65 and GlyR were by far the most frequently identified, leading to 3 immunologic groups: GAD65 antibodies (43.0%), GlyR antibodies (19.8%), and antibody negative (33.1%).

The clinical features associated with GAD65 antibodies were similar in many respects to those previously reported.[20,22-24] When compared with patients with GlyR antibodies or without antibodies, those with GAD65 antibodies were more likely to be female and have systemic autoimmune or endocrine disorders. The main neurologic differences among the 3 immunophenotypes depended on the relative frequency of symptoms included within the spectrum of PERM (hyperekplexia, myoclonus, brainstem, pyramidal, sensory, or autonomic dysfunction), which were mainly associated with GlyR antibodies, whereas the development of classic SPS or SLS with or without overlapping syndromes (eg, cerebellar ataxia, epilepsy) more frequently occurred with GAD65 antibodies. Patients without antibodies had a distribution of symptoms between those associated with GlyR and GAD65 antibodies. Although these differences were statistically significant, there was no clear syndrome-immunologic specificity, indicating that any form of SPSD can potentially occur with any of the 3 main immunologic groups considered here.

A frequent concern that arises in clinical practice is how frequently other antibodies that are less accessible in clinical laboratories (eg, DPPX, GABAaR, gephyrin) are missed because they are not tested for. The current data indicate that in our setting (a reference center for autoimmune and paraneoplastic disorders of the central nervous system) the frequency of antibodies other than GAD65 and GlyR is low. Indeed, only 5 patients (4.1%) had antibodies to amphiphysin, DPPX, or GABAaR, and none had antibodies to gephyrin, GlyT1, or GlyT2. These transporters were included because their mutations result in symptoms similar to those reported in mutations or autoimmunity to GlyR (hyperekplexia).[25] Compared with previous studies,[24,26] our findings reveal a larger group of seronegative patients (40 [33.1%] of 121 patients vs 16 [23.5%] of 68 patients[24] and 18 [18.2%] of 99 patients[26]). This finding is likely explained by the fact that 20

patients (16.5%) were referred to our center for assessment of novel or atypical antibodies after clinical or commercial antibody tests (mostly composed of GAD65, amphiphysin, or GlyR) tested negative. This referral pattern emphasizes even more the low frequency of DPPX and GABAaR antibodies among patients who test negative for the more common autoantibodies. The low frequency of amphiphysin antibodies has been reported in previous series.[27,28] Nevertheless, amphiphysin antibodies are important to consider in the paraneoplastic context, mainly breast and lung cancer.[27]

Detection of GABAaR antibodies in CSF or at high titers in serum (≥1/160) is associated with encephalitis with severe seizures or status epilepticus but without SPSD.[15] Therefore, detection of these antibodies only in serum and at low titers should be interpreted with caution. Similar caution should be considered for low serum titers of GlyR antibodies, which as reported here and in previous studies[29-31] occurred in 6% to 10% of controls without SPSD (eg, multiple sclerosis, cerebellar degeneration, or epilepsy). When CSF was available, none of these patients had GlyR antibodies in CSF, a finding that needs confirmation with a larger number of patients. In contrast, for GAD65 antibodies, we used 2 previously validated techniques (immunohistochemistry with rat brain and cell-based assay) that only reveal these antibodies if they are present at moderate to high titers, similar to those associated with neurologic disorders; titers equivalent to radioimmunoassay values of 2000 U/mL or less (seen in many patients with diabetes mellitus) are not detected with the techniques used here.[20,31]

A novel finding of our study is that the underlying mechanism (eg, presence and type of antibodies) but not the type of syndrome (eg, SPS, SLS, SPS-plus, or overlapping syndrome) was an independent predictor of outcome. For example, although at disease diagnosis patients with GlyR antibodies had more severe neurologic deficits than patients with GAD65 antibodies (and similar to antibody-negative patients), the outcome of patients with GlyR was better than that of patients with GAD65 antibodies. This finding could be explained by an early diagnosis in patients with GlyR antibodies (as shown in our study), which is likely owing to a more rapid and severe symptom onset (eg, SPS-plus or PERM) and the presence of CSF inflammatory changes when compared with patients of the other groups. Moreover, patients with GlyR antibodies were treated more aggressively (eg, first-line immunotherapy combined with second-line or long-term oral immunotherapy) than those of the other 2 groups, who more often received first-line immunotherapy and symptomatic treatment. On the other hand, disorders associated with antibodies to cell surface antigens, such as GlyR, are usually more responsive to immunotherapy than those associated with intracellular antigens, such as GAD65 or paraneoplastic antigens.

Our study has limitations related to its retrospective nature and possible selection of cases toward complex syndromes (with elements of PERM or overlapping syndromes). These limitations may explain the lower frequency of GAD65 antibody-positive cases compared with that of other series (52 [43.0%] of 121 patients vs 50 [73.5%] of 68 patients[24] and 79 [79.8%] of 99 patients[26]). Despite this, to our knowledge, the current series provides the most comprehensive autoantibody screening reported to date in SPSD. One could argue that using cultures of hippocampal neurons we missed antibodies directed to novel antigens restricted to the brainstem or spinal cord, a possibility to consider in future studies.

Conclusions

Several practical implications can be derived from this and previous studies.[32-34] First, SPSD is a complex group of disorders, with multiple autoantigens but 3 predominant immunophenotypes (GAD65 antibodies, GlyR antibodies, and antibody negative). Second, this immunologic characterization and the severity of symptoms are predictors of outcome. Third, although PERM predominantly occurs with GlyR antibodies, it can potentially be associated with other autoantibodies. This implication is important because, contrary to the concept that PERM carries a poor prognosis,[35] our findings indicate that this depends on the underlying immune response (worse in patients with GAD65 antibodies than antibody-negative patients and patients with GlyR antibodies). Fourth, DPPX and GABAaR are infrequent in SPSD; our data do not support upfront testing for these antibodies unless the clinical context (eg, gastrointestinal symptoms, hyperekplexia, encephalopathy for DPPX,[36] or prominent seizures for GABAaR) suggest their investigation.

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Supplementary Online Content

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eMaterial.

This supplementary material has been provided by the authors to give readers additional information about their work.

eMaterial

Patients excluded after identifying a final diagnosis other than SPSD

Patients with a final diagnosis of other disorders included, 6 cases with a functional disorder, 4 peripheral nerve hyperexcitability, 2 sporadic Creutzfeldt-Jacob disease, 2 amyotrophic lateral sclerosis, and one of each, tetanus, subacute combined spinal cord degeneration, primary progressive multiple sclerosis, Hashimoto encephalopathy, myotonia congenita, and myopathy.

Patients with immune mediated CNS disorders used as controls

Among the 195 patients with other immune mediated CNS disorders used as controls, 75 (44 with paired CSF) had GAD65 antibodies and symptoms other than SPS (cerebellar ataxia, epilepsy, or limbic encephalitis); 55 (30 with CSF) multiple sclerosis; 55 (49 CSF) encephalitis associated to neuronal-surface antibodies (NMDA-receptor, LGI1, or GABAb-receptor), and 10 anti-Hu associated paraneoplastic symptoms.

Antibody assays

Serum and CSF were examined for GAD65 and amphiphysin antibodies using frozen sections of paraformaldehyde (PFA) perfused sagittal sections of rat brain, as reported.[1] The presence of GAD65 antibodies was confirmed with cell-based assay (CBA) as described,[2] and amphiphysin antibodies were confirmed with immunoblot. GlyR and GABAaR antibodies were determined with live CBAs as reported.[3, 4] Antibodies to gephyrin were determined by CBA using HEK293 cells expressing (transfection ratio 1:1) cherry-labeled human Gephyrin and hPEM2 or collybistin, a protein necessary for membrane anchorage of gephyrin. HEK293 cells were fixed for 10 minutes in 4% PFA, permeabilized, and incubated with patients' serum (1:40) or CSF (1:5) overnight at 4ºC, followed by the secondary Alexa Fluor 488 goat anti-human antibody (1:1000, A11013 Molecular

Probes/ Life Technologies). Antibodies to the Glycine transporters 1 and 2 (GlyT1, GlyT2) were determined using HEK293 cells expressing myc-tagged human GlyT1 or GlyT2. Cells were incubated with patients' serum (1:40) or CSF (1:5) for 1 hour at 37ºC, fixed and permeabilized, and serially incubated with a myc-tag mouse monoclonal antibody (1:5000, 2276, Cell Signaling Technology, Inc.), the indicated anti-human secondary antibody, and Alexa Fluor 594 goat anti-mouse (11005, Molecular Probes/Life Technologies).

Additional antibodies against neuronal cell surface proteins were determined in serum and CSF using an immunofluorescence assay with dissociated rat hippocampal neuronal cultures as reported.[5] Positive cases were tested for antibodies to DPPX or other antigens (NMDAR, AMPAR, GABAbR, Caspr2, LGI1) by CBAs, as reported.[6] Results were photographed under fluorescence microscopy (Zeiss Axioimager M2) using Zeiss Axiovision software (Zeiss, Oberkochen, Germany).

Summary of 5 patients with antibodies other than GAD65 or GlyR

Five patients without GAD65 and GlyR antibodies had autoantibodies against other known antigens (2 amphiphysin, 2 GABAaR, and 1 DPPX). One of the two patients with amphiphysin antibodies had SPS with limbic encephalitis and breast cancer, and the other had SLS in association with breast cancer. The patient with DPPX antibodies developed SPS along with hyperekplexia, myoclonus, upper motor neuron dysfunction, sensory symptoms, orthostatic hypotension and prominent pruritus, all symptoms preceded by diarrhea and 15% weight loss. The 4 patients with GABAaR antibodies (2 of them with concurrent GAD65 antibodies) were young male (median 20 years, range 12-46) who developed classic SPS (n=2), SLS (n=1), and overlapping SPS with epilepsy (n=1, discussed above); these cases have been previously reported.[4]

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eTable 1. Clinical Features According to the Type of Syndrome

Clinical features	SPS N=50 (41%)	SLS N=24 (20%)	SPS-plus N=37 (31%)	Overlapping syndromes N=10 (8%)	p value
Female sex (%)	30 (60)	18 (75)	20 (54)	7 (70)	0.38
Age at onset, median years (IQR)	47 (38-56)	47 (45-58)	60 (44-66)	52 (28-63)	0.055
Delay to diagnosis, median years (IQR)	2 (1-5)	1 (0-3)	1 (0-2.5)	3 (0.5-5)	0.151
Systemic autoimmune disorders	26 (52)	13 (54)	13 (35)	4 (40)	0.51
Cancer	0 (0)	1 (4)	1 (3)	1 (10)	0.49
CMUA on EMG	21/32 (65.6)	10/19 (53)	17/28 (61)	4/5 (80)	0.49
CSF pleocytosis	4/24 (17)	0/11 (0)	10/28 (36)	1/6 (17)	0.017
CSF OCBs	7/23 (30)	1/13 (8)	9/24 (38)	1/3 (33)	0.17
Antibodies:					0.002
GAD65	27 (54)	10 (42)	7 (19)	8 (80)	
GlyR[a]	5 (10)	5 (21)	12 (32)	0 (0)	
Other	1 (2)	2 (8)	1 (3)	1 (10)	
Maximum mRS score, median (IQR)	4 (2.5-4)	3 (2-4)	4 (3-5)	4 (2-5)	0.002*
Symptomatic treatment	42 (84)	16 (67)	21 (57)	5 (50)	0.006
Type of immunotherapy:					0.009
Non-treated	10 (26)	6 (32)	1 (3)	1 (13)	
First line only	19 (50)	9 (47)	10 (31)	3 (37)	
First line and long-	5 (13)	1 (5)	10 (31)	0 (0)	

term oral					
First and second line	4 (10)	2 (11)	9 (28)	4 (50)	
Other	1 (3)	1 (5)	2 (6)	0 (0)	
Cases with follow-up	34 (68)	14 (58)	29 (78)	5 (50)	0.22
Follow-up period, median months (IQR)	17 (11-66)	54 (12-72)	18 (9-42)	24 (15-37)	0.54
Relapsing course	9 (26.5)	3 (21)	12 (41)	2 (40)	0.61
Final mRS score, median (IQR)	2 (1-3)	2 (1-3)	2 (1-5)	3.5 (2-5)	0.39
Change in mRS score, median (IQR)	1 (0 - 1)	1 (0.75 - 2)	2 (-1 - 3)	0.5 (-0.75 - 1)	0.51

CMUA: continuous motor unit activity; IQR: interquartile range; mRS: modified Rankin scale; OCBs: oligoclonal bands; SLS: stiff-limb syndrome; SPS: stiff-person syndrome; SPS-plus: progressive encephalomyelitis with rigidity, myoclonus, or other symptoms.

[a] Two additional patients had concurrent GAD65 and GlyR antibodies

* Mann-Whitney U test: maximum mRS SP-plus vs SPS p=0.001, SPS-plus vs SLS p=0.002, SPS-plus vs overlapping syndromes p=1

eTable 2. Clinical Features of the Main Immunologic Groups With EMG Findings Consistent With SPS (Continuous Agonist-Antagonist Motor Unit Activity)

Clinical features	**GAD65+ N=26 (54%)**	**GlyR+ N=6[a] (13%)**	**Antibody-negative N=16 (33%)**	**p value**
Female sex (%)	23 (88)	1 (17)	8 (50)	0.001
Age at onset, median years (IQR)	54 (45-65)	52 (33-62)	47 (36-63)	0.38
Delay to diagnosis, median years (IQR)	2 (1-4)	0 (0-2)	1.5 (0.5-5)	0.10
Syndrome:				0.005
SPS	13 (50)	0 (0)	7 (44)	
SLS	7 (27)	0 (0)	2 (12)	
SPS-plus	3 (11)	6 (100)	6 (38)	
Overlapping syndromes	3 (11)	0 (0)	1 (6)	
Hyperekplexia	9 (35)	5 (83)	4 (25)	0.021
Myoclonus	2 (8)	4 (67)	3 (19)	0.014
Brainstem	2 (8)	6 (100)	2 (13)	<0.001
Pyramidal	1 (4)	4 (67)	5 (31)	0.001
Sphincter	1 (4)	3 (50)	5 (31)	0.039
Sensory	0 (0)	3 (50)	2 (13)	0.013
Autonomic	2 (8)	4 (67)	2 (13)	0.034
Insomnia	2 (8)	4 (67)	1 (6)	0.013
Systemic autoimmune disorders[b]	18 (69)	3 (50)	6 (38)	0.25
CSF pleocytosis	2/11 (18)	2/6 (33)	1/11 (9)	0.029
CSF OCBs	6/14 (42)	1/6 (17)	0/6 (0)	0.016
Maximum mRS score, median (IQR)	4 (3-4)	5 (4-5)	4 (3-5)	0.012*
Symptomatic treatment	26 (100)	4 (67)	12 (75)	0.007
Type of immunotherapy:				0.05
Non-treated	5 (19)	0 (0)	2 (13)	
First line only	12 (46)	0 (0)	6 (38)	
First line and long-term oral	4 (15)	3 (50)	2 (12.5)	
First and second line	3 (12)	3 (50)	0 (0)	
Other	1 (4)	0 (0)	1 (6)	
Cases with follow-up	25 (96)	6 (100)	11 (69)	0.034
Follow-up period, median months (IQR)	32 (11-72)	27 (9-129)	48 (12-75)	0.91
Relapsing course	11 (44)	3 (50)	5 (45)	1
Final mRS score, median (IQR)	2.5 (1-3)	1.5 (1-5)	2.5 (1.5-5)	0.68

Improvement:				0.70
Treated	16/20 (80)	4/6 (67)	5/9 (56)	
Non-treated	2/5 (40)	0 (0)	1/2 (50)	
Change in mRS score, median (IQR)	1 (0 - 2)	3 (-0.25 - 4)	0.5 (-0.25 - 2)	0.24

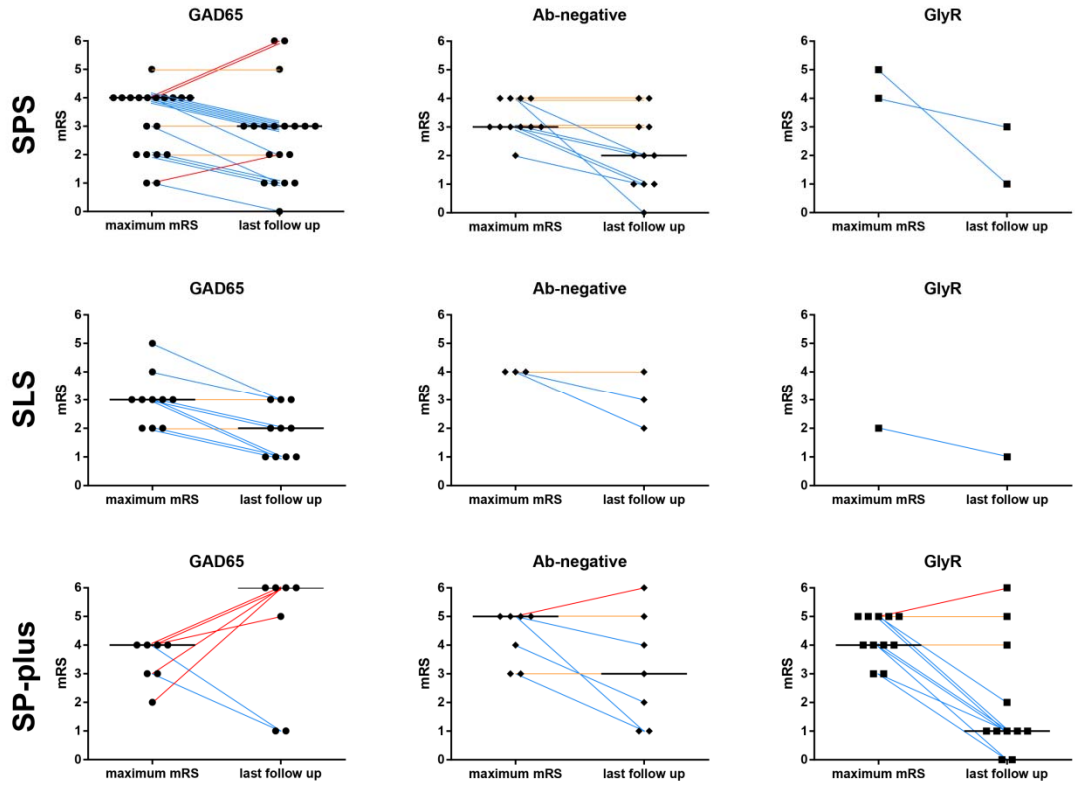
IQR: interquartile range; mRS: modified Rankin scale; SLS: stiff-limb syndrome; SPS: stiff-person syndrome; SPS-plus: progressive encephalomyelitis with rigidity, myoclonus, or other symptoms.

[a] Excludes 1 patient with concurrent GAD and GlyR antibodies

[b] 27 patients (56%): including 6 with type I diabetes mellitus, 6 thyroiditis, 1 celiac disease, 1 psoriasis, 1 vitiligo, 1 Raynaud syndrome, and 11 had thyroid, ANA, dsDNA or gastric parietal cell antibodies

* Mann-Whitney U test: maximum mRS score GAD65+ vs GlyR p=0.002, GAD65+ vs Ab-neg p=0.22, GlyR+ vs Ab-neg p=0.037

eFigure. Outcome According to the Clinical Syndrome and Immunologic Group



Patients' maximum mRS score compared with the score at the last follow-up according to the main syndromes: first row stiff-person syndrome (SPS), second row stiff-limb syndrome (SLS), and third row stiff-person syndrome plus (SPS-plus); and according to the immunologi group: first column GAD65 antibodies, second column antibody-negative (Ab-neg), and third column GlyR antibodies. Improvement for each individual patient is depicted in blue, deterioration in red, and no change in orange. The median mRS is provided as a horizontal black line (only provided if $n \geq 5$). In multivariable analysis on group level, presence of GAD65 antibodies was associated with less favorable outcome (Table 2).

Discussion

Since its description in 1988 by Solimena and coworkers,[8] GAD-ab have become an excellent biomarker of autoimmunity in certain neurological disorders. Initial research established determinant differences between antibodies from patients with autoimmune diabetes, where GAD is still the major autoantigen, and neurological disorders.[12,17,24,91,132,140] This means that pathomecanisms, clinical course and potential immunotherapeutic strategies cannot just be extrapolated from scientific achievements in the field of T1DM. Research focused in neurological disorders is then essential, and at the same time is a challenge due to the low frequency of these diseases.

Previous work of this group, which also led to a thesis in the past, resulted fundamental in the contribution to the knowledge of GAD-autoimmunity involvement in CNS disorders. Those efforts served to provide guidelines on the diagnostic value of high titres of GAD-ab in patients with neurological syndromes. In this sense, it is noteworthy the first descriptions of cerebellar ataxia associated to GAD-ab[59] and the identification of partial forms of SPS[33] that helped to complete the clinical spectrum of GAD autoimmunity, or the study of the prevalence of GAD-ab in epileptic syndromes and the identification of specific clinical phenotypes among epileptic patients[71,73]. Despite these observations, major knowledge gaps remained to be elucidated.

One of the most intriguing questions is why some patients develop a stiff-person syndrome, and others present with a cerebellar dysfunction, for example. In ***Paper I*** we addressed this question with a very comprehensive study of the largest cohort of GAD-related neurological syndromes described so far. Despite this strength, we did not find relevant

differences in the humoral immune-response trough the diverse neurological syndromes. One explanation is that our approach was not powered enough in some aspects. The epitope analysis was performed using clones that spanned the whole sequence of the GAD65, but in 3 large parts corresponding to the 3 functional domains of the enzyme. That might completely overlook a small linear epitope within a functional domain or a relevant conformational epitope dependent of the spatial relation of 2 or more domains. A contemporary work of another independent group using an immunoprecipitation assay with 3 constructs, also found that the main immunodominant region of the epitope, was located in the catalytic core in all the GAD65-ab containing sera with independence of the clinical syndrome.[141] But unless a more accurate experiment is done, the hypothesis of different epitope specificity within the GAD65 isoform cannot be definitely ruled out. Other explanation is that these differences actually do not exist. Indeed, there is evidence that other promoting factors are driving the disease. The sole presence of circulating antibodies against GAD65 is not apparently sufficient to develop a neurological disorder. The long latency between GAD-ab detection and development of limbic encephalitis in *Case Report I* is an example; although high serum titres in this case could be the consequence of the polyglandular autoimmune syndrome, which is the associated endocrine disorder that harbours higher levels of GAD-ab. Another piece of evidence is the precedent of 2 patients with SPS who underwent autologous hematopoietic stem cell transplantation and achieved sustained clinical remission more than 2.5 and 4.5 years after the procedure, despite the persistence of GAD65-ab.

The comprehensive screening that we accomplished shows that the analysis of CSF is essential in these neurological conditions, and that no

definitive conclusion about the role of GAD-ab can be achieved from the study of serum samples. The specific intrathecal synthesis had been previously shown, indicating that the presence of antibodies in the CSF is not a phenomenon of passive antibody transfer from serum. But results emerging from this thesis point to a specific intrathecal maturing of the humoral response. On one hand, the GAD65 epitope recognition is generally broader in CSF that in serum. In 46% of patients, serum GAD65-ab recognized only one domain, whereas 74% of CSF GAD65-ab recognized all three domains. This indicates an intramolecular B cell epitope spreading driven intrathecally. The epitope spreading can potentially occur through many mechanisms, including interactions with T cells, endocytic processing of antigens, and somatic hypermutation in the B cell. While the presence of auto-antibodies may antedate in years the clinical presentation of autoimmune diseases, intramolecular epitope spreading has been demonstrated to be one of the events that precede the development of symptoms in entities like T1DM, pemphigus, systemic lupus erythematosus or experimental allergic encephalomyelitis (the murine model of multiple sclerosis).[142] In an autoimmune encephalitis mediated by antibodies against LGI1, somatic hypermutation rates point to a CSF antigen-driven activation of clonally related B cells that shape the intrathecal immune repertoire.[143] On the other hand, the frequency of antibodies against GAD67 is significantly higher in CSF than in serum. This fact is important because GAD67, the other isoform of the enzyme, has been historically neglected in the study of these diseases. A relevant circumstance is that GAD67 is mainly expressed in neurons, and not in beta pancreatic cells. The low frequency of GAD67-ab in patients with T1DM seems to be explained by a phenomenon of cross-reactivity due to the sequence similarities between

both isoforms.[131] However, we demonstrated that in neurological conditions, the prevalence of GAD67-ab is 100% in CSF. This, along with the report of a patient with cerebellar ataxia and GAD67-ab but not GAD65-ab (*Case Report II*), suggests that GAD67 deserves further investigations to determine its role. Whether their presence is the sterile result of the epitope spreading, or they have a pathogenic role is presently unclear. Nevertheless, the intracellular distribution of GAD67 associates the same theoretical concerns that are still unsolved for GAD65-ab, regarding the pathogenic potential role. In our set of experiments, we were not able to demonstrate the internalization of antibodies contained in CSF samples (GAD65 and GAD67) into live rat hippocampal neurons with the same procedure that clearly demonstrated the internalization of NMDAR IgG. At least, GAD67-ab have a potential role as diagnostic biomarker, since they may be detected even in the absence of GAD65-ab.

Despite the absence of specific differences in the humoral response linked to the diverse phenotypic presentation, the following question that we tried to address was if the clinical course is similar among the different GAD-associated neurological syndromes. In *Paper II* we focused on patients with cerebellar ataxia to determine the long-term outcome, an issue not well known. The frequency of disabled patients due to their neurological condition after years of follow-up was higher than an autonomous outcome. This was associated logically to a lower disability at the time of diagnosis, reflecting probably a natural lower aggressiveness of the disease in some patients, but also to the effect of immunotherapy. A sustained improvement after treatment was reported in 35% of treated patients, and the frequency of this beneficial effect was higher, although not exclusive, in those patients with a subacute onset. Independently of the therapy, the

subacute onset emerged as a predictive factor of a better outcome in the multivariate analysis. These findings, along with the absence of differential brain atrophy in MRI, supports the hypothesis that it may exist an initial phase of the disease where the neurological symptoms have a functional component. And this effect is potentially reversible before definite structural damage is established. Immunotherapy is, then, indicated in every patient with GAD-associated cerebellar ataxia. The limitations of the study preclude revealing any significant evidence regarding the type or the duration of immunotherapy, but the delay to treatment appeared as a prognostic factor. This data indicates the convenience of treating as soon as possible these patients, and, therefore, the importance of prompt recognition of the syndrome. The cerebellar dysfunction is difficult to distinguish from other sporadic cerebellar syndromes of autoimmune aetiology like a paraneoplastic cerebellar degeneration, especially if the neurological symptoms progress rapidly in a subacute manner. Interestingly, our investigations led also to important clinical clues to recognize these patients. A proportion of patients presented neurologic symptoms antedating the development of a full cerebellar syndrome. The most frequent picture was a fluctuating vertigo and this could happen up to 26 months earlier. A second clue confirmed in this series is that the coexistence of partial manifestations of the SPS spectrum are not infrequent and may present simultaneously; EMG may be useful in cases where symptoms are not evident. A third clinical aspect is the known co-existence of other organ-specific autoimmune disorders in the context of GAD autoimmunity, and the detection of concomitant T1DM or thyroiditis (clinical or subclinical) may help to suspect this specific autoimmunity. These features should raise the clinical suspicion and the subsequent

search for GAD-antibodies. The detection of antibodies is assessable by commercial tests, broadly available in non-research centres. As we have learned, there might be some cases where isolated GAD67 immunoreactivity may be overlooked with commercial tests. So, to send the samples to a reference laboratory is a good practice, especially when a first screening is negative.

Other important challenge that clinicians have to face is when to suspect a paraneoplastic origin in cases of neurological symptoms, high-titre of GAD65-ab, and the absence of onconeuronal antibodies. Due to the low prevalence reported, the systematic screening may unjustifiably increase the cost in the health care of these patients, and at the same time the likelihood of iatrogenicity. Our investigations published in *Paper III* resulted in important clues to recognize patients with a hidden tumour, and the relevance of these findings were highlighted with an award in the European Academy of Neurology, and an editorial in the journal where the study was published. The clinical presentation in paraneoplastic cases moves away from the most typical presentation of GAD-related-neurological syndromes: a woman under 50 years old who develops a SPS or a CA with an insidious onset. In contrast, patients with an associated tumour were older, males or presented other neurological syndromes (either classic paraneoplastic syndromes like limbic encephalitis, or atypical syndromes like brainstem dysfunction). Considering only this clinical features at presentation, 13 out of 15 patients from our series could be suspected to be paraneoplastic. Moreover, we found a significantly higher proportion of antibodies against neuronal surface antigens in patients with tumours. Hence, a comprehensive immunological test including the search for additional antibodies besides GAD65-ab may help to increase the clinical

suspicion. In our cohort, the type of tumour aggregated around thymomas, and tumours with neuroendocrine tissue expression (SCLC, pancreatic). These tumours are able to trigger a paraneoplastic pathogenesis through 2 mechanisms: a) an ectopic expression of neural antigens, demonstrated by immunohistochemistry of the tumoral tissue in 3 cases; b) loss of natural immune tolerance to self, typical in disorders of central lymphoid organs like thymus. Altogether, these reasons support a true paraneoplastic origin in most patients. Only 2 patients from the series were clinically indistinguishable from a non-paraneoplastic GAD-associated neurological syndrome, and the level of certainty of a paraneoplastic origin in both cases is far from definite, since they could correspond merely to an epidemiological association. These patients were females with SPS or slow CA, breast cancer, and GAD65-ab as the only biomarkers of autoimmunity.

In the last objective of this thesis, addressed in *Paper IV*, we investigated the immunological spectrum in SPSD in the era of the neuronal cell surface autoantibodies, and the results led to the conclusion that immunological status may help to better classify these patients. Although being a large cohort, it may have an important bias of referral, because we are a reference center for autoimmune and paraneoplastic disorders of the central nervous system. Clear clinical cases ("classic SPS") or those GAD-ab-positive, which are broadly accessible in clinical laboratories, may be under-represented. This forces to interpret with caution the relative frequency of immunological or clinical groups, but does not invalidate the relation among immunostatus and clinical features. Among 121 patients, only 41% had typical SPS and only 60% had a typical EMG. This highlights the limitations of the actual diagnostic algorithm, which relies on clinical and electrophysiological criteria. The only recognized biomarker so far,

GAD65-ab, was present only in 54% of patients with typical syndrome or EMG, and this frequency decreases to 43% of the entire cohort when considering more challenging cases. A comprehensive screening did not identify a novel diagnostic biomarker; GlyR-ab (not coexisting with GAD-ab) were positive in around 20%, and seronegative is still an important group (33.1%). This last group is interesting. After excluding 20 patients whose serum or CSF was referred to our laboratory and finally had an alternative diagnosis, still 40 remained fulfilling clinical suspicion of SPSD. Indeed, at least half of them had typical SPS or a compatible EMG. A high proportion (30%) had other organ-specific autoimmunities like in GAD autoimmunity; 80% received some kind of immunotherapy, and in those cases 60% improved a median of 1 point in mRS scale. Considering these findings, the probability of a different etiology in these patients is low, and we hypothesize that the pathomechanism in many of them is also an autoimmune dysfunction of the inhibitory synapse with a less evident humoral autoimmunity. One possible explanation is the aggregation is this group of cases with a positive CSF and a negative serum. Three of 26 GAD-ab-positive cases in this cohort were only positive in CSF, and 16 patients in the seronegative group did not have a CSF available sample. Other possibility is a technical problem regarding sensitivity of the tests used. Whereas in previous studies we proved that immunohistochemistry detects high levels of GAD65-ab (over 2000 U by RIA), the sensitivity of our cell-based assay for lower titers has not been specifically investigated and could be unable to detect lower levels of GAD-ab. Nevertheless, the contribution of this work is the confirmation that SPSD is a heterogeneous disorder from the immunological point of view and that the immunostatus does not unequivocally correlates with the clinical presentation (the

neurological syndrome). However, the most remarkable value of antibody testing is that the presence and type of autoantibodies are predictors of outcome. In contrast, the type of syndrome did not predict outcome. Patients with GlyR-ab, despite a more severe clinical presentation, had a greater degree of improvement than those with GAD-ab or without antibodies. GAD-ab predicted the worse outcome in this cohort of SPSD, consisting in a higher degree of disability at last follow-up visit along with the absence of substantial improvement after immunotherapy.

In light of these results, the screening of antibodies in SPSD should be incorporated to the initial evaluation of these patients. The magnitude of the prognostic value can be confirmed only with a prospective determination of the antibodies, to avoid the potential selection bias of difficult or severe cases.

Summary of the contribution of this thesis and final thoughts

We investigated comprehensively the immune-response in the largest cohort of neurological syndromes associated with GAD-ab ever published, and additionally we focused on subgroups of patients with a clinical gap. Our results confirmed that autoimmunity regarding the humoral response is similar among different neurological syndromes, and that GAD-ab is still the most important biomarker in these diseases. The research focus in the future should come back to this antigen to clarify the role of these antibodies in a complex disorder of the immune system, and to design strategies to modulate this aberrant autoimmunity once developed. In the case of T1DM, the reference of GAD-associated disorder, no single intervention towards immunosuppression convincingly preserves the

residual β-cell function present at the time of clinical diagnosis.[144] In contrast, interventions directed to re-establish the balance between the harmful and the regulatory arms of the autoimmunity seem more promising. The expansion of disease-specific regulatory T cells using relevant autoantigens like GAD have shown hopeful results in preclinical models of several autoimmune diseases, including a T1DM murine model.[145] Future trials will clarify whether this is the best approach to treat these GAD-associated neurological disorders.

For the daily practice, our investigations enlighten the value of these antibodies in SPSD, and come up with clinic-immunological clues to early recognize patients with GAD-associated cerebellar ataxia, and paraneoplastic neurological syndromes. Both groups of patients are challenging from a diagnostic point of view, and their outcomes partially reside in the possibility of a prompt treatment.

Conclusions

1. The presence of additional antibodies against antigens of the inhibitory synapse or a different reactivity against particular GAD isoforms or sites of GAD65 do not explain the diversity of the clinical phenotype in non-paraneoplastic neurological syndromes associated with GAD-ab

2. The immunological response against GAD is different in serum and CSF, indicating a process of antigen-driven intrathecal maturation in patients with non-paraneoplastic syndromes

3. Patients with cerebellar ataxia and GAD-ab may respond to immunotherapy, and maintain good functional status at long-term. Early initiation of treatment likely offers a greater chance of improvement

4. Neurological syndromes with paraneoplastic criteria in the context of GAD autoimmunity have a different clinical presentation and humoral immunity profile. Patients presenting neurological syndromes not typically associated with GAD-ab should be screened for an underlying cancer

5. Among patients with stiff-person spectrum disorders, the immunological classification is an independent predictor of outcome. Those patients with GAD-ab have worse prognosis than antibody-negative patients and patients with GlyR-ab.

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Annex

Case Report I

Long latency between GAD-antibody detection and development of limbic encephalitis – a case report.

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CASE REPORT Open Access

Long latency between GAD-antibody detection and development of limbic encephalitis – a case report

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Abstract

Background: In the pathogenesis of limbic encephalitis other promoting factors besides the pure existence of autoantibodies are increasingly discussed to play a significant role. This is to our knowledge the first described patient in whom the presence of autoantibodies precedes the manifestation of limbic encephalitis for many years.

Case presentation: At the age of 38 years, in the serum of a patient with polyendocrine autoimmunity high titers of cytoplasmic islet cell antibodies and of anti-glutamate decarboxylase (GAD) 65 antibodies were observed as an incidential finding, GAD67 antibodies were negative at that time. After a latency of 18 years, she manifested with refractory temporal lobe epilepsy most likely due to autoimmune limbic encephalitis. After epilepsy onset, the patient underwent magnetic resonance imaging (MRI), electroencephalography, cerebrospinal fluid (CSF), serum and neuropsychological investigations during a follow-up period of 8 years. A pharmacoresistent epilepsy with seizure onset from the right temporal lobe and declarative memory deficits were observed affecting primarily the recall of verbal informations. MRI showed a slightly increased signal in the right amygdala without progression. GAD antibodies could be detected in serum (titre 1: 1000) and CSF (titre 1:1) by immunofluorescence. Both, GAD65 and GAD67 antibodies were observed in cell-based assays.

Conclusions: It can be assumed that in addition to a pre-existing systemic T-cell response associated with the longstanding polyendocrine autoimmunity, a delayed intrathecal autoimmunity developed leading to limbic encephalitis. This change might be reflected by the development of GAD67 antibodies in our patient. Besides the contribution of this case report to a better understandig of the pathomechanisms for the development of central nervous system (CNS) autoimmunity, it also has a clinical impact as early treatment of GAD antibody-associated CNS disorders has a better prognosis. Therefore, vigilance for symptoms indicating GAD antibody-associated CNS autoimmunity is mandatory in patients with GAD antibody-associated endocrine dysfunction.

Keywords: Clinical manifestation, GAD antibodies, Limbic encephalitis, Pathogenesis

Background

Limbic encephalitis describes a heterogenous spectrum of neurological disorders characterized by mostly subacute onset and progressive mnestic deficits, epileptic seizures and psychiatric disturbances such as depression and psychosis. A variety of autoantibodies against brain antigens has been detected in association with limbic encephalitis. These include onconeuronal antibodies against intracellular proteins (Hu, Ma2, CV2) in the context with malignant tumors [1] and a multitude of other distinct pathogenic autoantibodies against surface protein receptors in the absence or presence of tumors [2] .

Anti-glutamic acid decarboxylase (GAD) antibodies occupy an intermediate position, as they are directed against an intracellular antigen but are not associated with malignant tumors in the majority of cases [2]. Two distinct GAD isoforms exist in humans, GAD65 and GAD67, which are encoded by different genes and share approximately 75 % amino acid sequence identity [3]. Both isoforms are expressed in islet cells and GABAergic neurons, although GAD65 at higher levels. The molecular nature of GAD antibodies is partly distinct in different clinical

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conditions [4]. Patients with GAD antibodies manifest with different clinical disorders, either with or without neurological dysfunction. Non-neurological disorders comprise type 1 diabetes mellitus alone or polyendocrine autoimmunity with or without diabetes [5, 6] while neurological disorders associated with GAD antibodies include stiff person syndrome (SPS), cerebellar ataxia and limbic encephalitis sometimes presenting as therapy refractory temporal lobe epilepsy [7, 8]. While GAD antibodies in autoimmune endocrinopathies recognize conformational epitopes on GAD65 and the infrequent recognition of GAD67 isoform is assumed as cross-reactivity phenomenon [9], the antibodies in SPS and cerebellar ataxia recognize linear epitopes on GAD65 so they can be detected by Western blotting and frequently recognize GAD67 [10, 11, 12, 13]. Whereas a direct pathogenic mechanism of autoantibodies was demonstrated for antibodies to the N-methyl-D-aspartate receptor (NMDAR antibodies) [14] and can be assumed for limbic encephalitis associated with others surface protein autoantibodies [2], in cases with antibodies against intracellular antigens including GAD antibodies, the toxic effect on the central nervous system (CNS) is more probably mediated by cytotoxic T cells [2]. However, even in autoimmune encephalitis associated with NMDAR antibodies, it has been recently shown that an intrathecal antibody synthesis can persist for a long time after remission of acute NMDAR encephalitis [15]. Thus, other promoting factors seem to be essential to establish the clinical phenotype. Blood brain barrier function and antibody affinity have been discussed as such further important promoting factors.

Here, we report the first patient with limbic encephalitis associated with antibodies to GAD in whom these antibodies had already been detected 18 years *before* the patient became symptomatic with epileptic seizures and mnestic deficits. This case report adds to the medical literature that in longstanding polyendocrine autoimmunity a delayed intrathecal spread is possible leading many years later to limbic encephalitis.

Case presentation

At the age of 38 years, the female patient participated as a healthy control in scientific studies concerning cytoplasmic islet cell antibodies (ICA) in insulin-dependent diabetes using an indirect immunofluorescence tests on cryostat sections of human pancreas [16–18]. At that time she neither suffered from diabetes mellitus nor presented with any neurological symptoms. However, at the same time, she was diagnosed for autoimmune thyroiditis with high-titre antibodies to thyroid microsomal antigens (although hypothyreoidism had already been known for several years and treated with L-thyroxin), she had vitiligo and antibodies to gastric parietal cells as well as antibodies to the intrinsic factor. At that time - as an incidential finding - high titers of ICA (reportedly >1:128, immunefluorescence on human pancreas) and GAD antibodies were observed in the patient's serum. The GAD antibodies were directed to the GAD65 antigen only. Antibodies to GAD67 were negative at that time [5]. The ICA reactivity could not be eliminated by preincubation with GAD65 indicating additional antigens against which the ICA was directed [17].

At the age of 56 years, the patient presented with first epileptic seizures. Seizure semiology consisted mainly in simple partial seizures with tightness in the chest accompanied by anxiety resembling angina pectoris. These seizures lasted for 0.5 to 3 min. During most of these seizures, she was adequately responsive to verbal and non-verbal commands. Sometimes, there was a transition to complex partial seizures of temporal semiology. Reportedly, she was unresponsive and appeared helpless. Moreover, she showed fumbling manual automatisms for about 2 to 3 min. The patient had amnesia for these episodes. Additionally she reported acoustic hallucinations in terms of a perception of music/melodies. Generalised tonic-clonic seizures never occurred. Seizure frequency was several simple partial and complex partial seizures per month. Simultaneously with epilepsy onset, the patient complained of considerable memory impairment. A first neuropsychological examination revealed significant episodic memory deficits that affected primarily the recall of verbal information. The figural episodic memory as well as primary verbal memory and verbal fluency were only slightly reduced. Information processing speed, divided and selective attention were in the normal range. Initial cerebrospinal fluid (CSF) examination at the age of 56 was normal (0 leucocyte, total protein 324 mg/l, lactate 1,6 mmol/l, oligoclonal and oligoclonal IgG n serum and CSF negative).

In the cerebral magnetic resonance imaging (MRI) performed three years later, a slight T2-hyperintensity of the right amygdala without increased volume was observed.

At the age of 60, video-electroencephalography (EEG) monitoring was performed. Interictal epileptiform discharges were seen in the right temporo-anterior region. One habitual complex partial seizure could be registered with EEG onset also over the right temporal lobe. In a neuropsychological follow-up performed, a substantial worsening of the figural episodic memory performance was found, whereas episodic verbal memory and verbal fluency presented nearly normal. Repeated CSF analysis at the age of 60 again revealed a normal cell count (1 cells/μl), CSF/blood barrier function (total protein 271 mg/l, CSF/serum albumin ratio 3.0 x 10^{-3}) and absent intrathecal immunoglobulin synthesis. However, GAD antibodies were highly positive in both serum and CSF when tested by a GAD65 ELISA (serum 15.800 U/ml,

CSF 48 U/ml) as described [7] or using immunofluorescence on primate cerebellum (serum 1:1000, CSF 1:1) (Fig. 1a-c) and rat brain (serum 1:64,000, CSF 1:8) [7]. GAD antibodies in serum recognized a linear GAD65 epitope, as suggested by the positive reactivity in immunoblots using recombinant GAD65 protein [13]. Concomitant antibodies against GAD67 (serum 1:40, CSF 1:5) were found in serum and CSF using a cell-based assay [19]. Indirect immunofluorescence using both rat brain and primate cerebellum, pancreas and gut did not reveal the presence of antibodies at relevant titres to neuronal antigens other than GAD. Specific subtests could exclude NMDAR, CASPR2, LGI1, AMPAR, $GABA_BR$ and $GABA_AR$ antibodies in serum and CSF and GlyR, Aquaporin 4, Hu, Yo, Ri, CV2, Tr, Ma1/2, Zic4 antibodies in serum.

In the following years, the epilepsy took a pharmacoresistant course. The patient was treated by levetiracetam, lamotrigine and zonisamide without relevant improvement. The MRI abnormality remained unchanged over an observation time of 3 years (Fig. 1d). A further neuropsychological assessment at the age of 62 revealed again mnestic deficits mainly concerning the recall of verbal episodic information and – to a less severe degree - the primary verbal memory while non-verbal memory functions had improved. Moreover, the patient presented with deficits in the selective attention and with emotional lability as well as moderate depressive symptomes. However, during the course of the disease the patient remained able to work. At the age of 63, the patient received two cycle of high-dose methylprednisolone (1 g /daily for 5 consecutive days every four months). In parallel, the seizure frequency decreased considerably to less than once per month although in parallel her antiepileptic therapy was complemented by perampanel.

Until present, the patient has not developed diabetes mellitus.

Based on the clinical findings of late-onset temporal lobe epilepsy with a pharmacoresistant course, impairment of both, verbal and nonverbal declarative memory, a slight increase of signal intensity in the right amygdala without hippocampal atrophy or sclerosis and high GAD65 and positive GAD67 antibodies in serum and CSF, a GAD antibody-associated limbic encephalitis was diagnosed.

Conclusion

Initially, GAD antibodies in our patient were only associated with endocrine autoimmunity for many years. Thus, our case illustrates that besides the pure presence of the autoantibodies other promoting factors must play a role in the pathogenesis of limbic encephalitis. Whereas complete recovery from symptoms of autoimmune encephalitis has

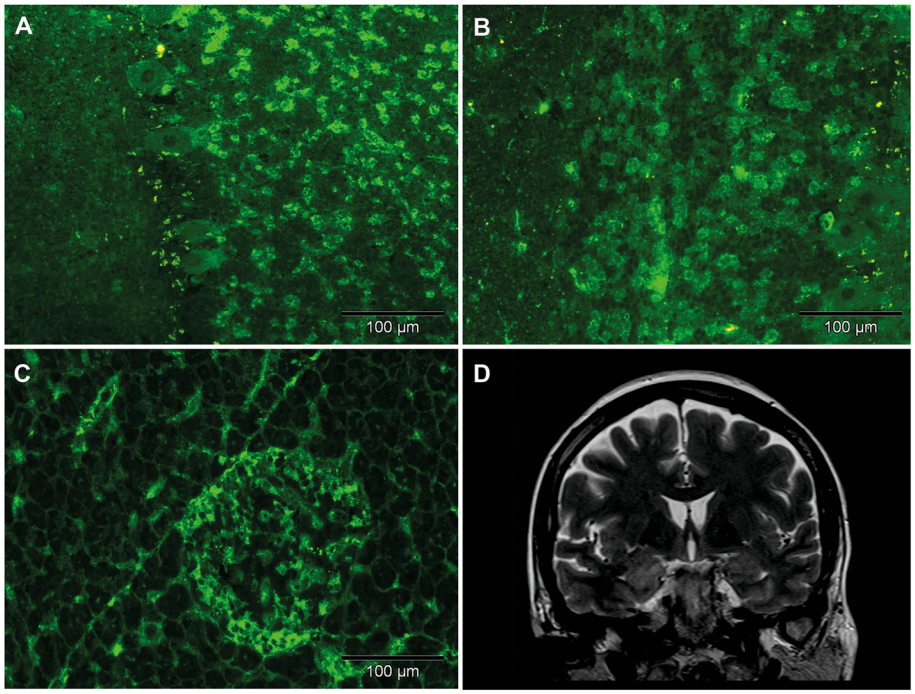

Fig. 1 Immunofluorescence findings and magnetic resonance imaging in our case of GAD antibody-associated limbic encephalitis. **a**-**c** Micrographs showing immunofluorescence using the patient's serum diluted 1:100 (**a**/**c**) or CSF diluted 1:1 (**b**) on primate cerebellum (**a**/**b**) or pancreas (**c**) (bar = 100 µm). Note the typical fluorescence pattern for GAD antibodies with prominent fluorescence in the granule cell layer and a subset of islet cells and less prominent signal in Purkinje cells. **d** T2-weighted magnetic resonance imaging demonstrates a slight signal hyperintensity of the right amygdala/anterior hippocampus compatible with the diagnosis of limbic encephalitis

been described despite the persistence of autoantibodies [15], our patient is to our knowledge the first in whom the presence of autoantibodies preceded the manifestation of limbic encephalitis for many years. Although autoimmune encephalitis in terms of GAD autoimmunity cannot be proven in our patient, there are many arguments in favor of this hypothesis. First, high antibody levels were found in the range previously described associated with GAD antibody-associated neurological syndromes [7], which detected a linear epitope as found in other GAD antibody-associated CNS disorders [13]. Second, GAD67 antibodies were positive in the CSF, a finding invariably present in patients with GAD antibody-associated disorders of the CNS [19]. Third, the clinical course with prominent pharmacoresistant epilepsy and less apparent psychiatric and cognitive disturbances is typical of GAD antibody-positive limbic encephalitis [8]. Fourth, in cranial MRI a slight increase of signal intensity was seen in the right amygdala without enlarged volume or hippocampal sclerosis, which remained without progression over three years. Thus, a low-grade brain tumor, mesial temporal sclerosis or a dysplasia is unlikely to exist in this case.

How could the long latency between the appearance of GAD antibodies and the clinical manifestation of limbic encephalitis be explained? In NMDAR encephalitis, in contrast to GAD antibody-associated syndromes, the NMDAR antibodies play a pathophysiologically relevant role [14]. Here, a spread from systemic to intrathecal autoimmunity against NMDARs is hypothesized to trigger the onset of the encephalitis [20]. In patients with antibodies against intracellular antigens, including those with GAD antibodies, a higher CD8/CD3 ratio and more frequent appositions of granzyme-B(+) cytotoxic T cells to neurons were found compared to the patients with surface antigens [21]. In analogy with NMDAR encephalitis, however, it can be assumed that in addition to the pre-existing systemic T cell response associated with the longstanding polyendocrine autoimmunity in our patient, a delayed intrathecal autoimmunity developed leading to limbic encephalitis. This change might be reflected by the development of GAD67 antibodies in our patient. Moreover, a (transient) blood–brain barrier disruption might have played an important part in the reported patient. Alternatively, the epilepsy may not have occurred immediately after tissue damage but after a longer latency period in which remodelling of synapses finally lead to epileptogenesis similar to hippocampal sclerosis.

In conclusion, our case shows that systemic GAD autoimmunity, even after many years, can spread to the CNS. Moreover, our case underlines that the presence of one or more autoimmune disorders is an indicator that epilepsy may be of autoimmune origin [22]. As early treatment of GAD antibody-associated CNS disorders has a better prognosis [19], vigilance for symptoms indicating GAD antibody-associated CNS autoimmunity is mandatory in patients with GAD antibody-associated endocrine dysfunction.

Consent

Written informed consent was obtained from the patient for publication of this Case report and any accompanying images. A copy of the written consent is available for review by the Editor of this journal.

Abbreviations

CNS: Central nervous system; CSF: Cerebrospinal fluid; EEG: Electroencephalography; GABA: Gamma-amino-butyric acid; GAD: Anti-glutamate decarboxylase; ICA: Cytoplasmic islet cell antibodies; MRI: Magnetic resonance imaging; NMDA: N-methyl-D-aspartate; NMDAR: N-methyl-D-aspartate receptor; SPS: Stiff person syndrome.

Competing interests

Jan Lewerenz received speaker's honoraria by Euroimmun, Lübeck, Germany. The other authors have no competing interests to disclose.

Authors' contributions

SF is the corresponding author. She developed the concept and has written the first draft of the case report and has treated the described patient as an epileptologist for several years. IU has performed the neurophsychological investigations in the patient over 7 years. HA and AS established and performed the ELISA and Western Blot for detection of GAD65 antibodies, the cell-based assay for GAD67 antibodies. WAS and his group have performed the first investigations of islet cell antibodies and GAD antibodies in the described patient. He, as an endocrinologist, has given substantially scientific input in this manuscript. JL has collected and evaluated the CSF results and the immunofluorescence on primate cerebellum and colon for detection of GAD antibodies in serum and CSF. All authors contributed to the final draft of the manuscript.

Authors' information

Not applicable.

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Not applicable.

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Case Report II

Cerebellar ataxia and autoantibodies restricted to glutamic acid decarboxylase 67 (GAD67)

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Short Communication

Cerebellar ataxia and autoantibodies restricted to glutamic acid decarboxylase 67 (GAD67)

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ABSTRACT

Cerebellar ataxia is one of the most frequent syndromes associated with autoantibodies against glutamic acid decarboxylase (GAD-ab). Antibodies recognize the isoform GAD65, which is the standard biomarker, but additional immunoreactivity against GAD67 is found in high proportion of patients with GAD-ab-associated neurological disorders. We describe the case of a 59-year-old woman who presented with pancerebellar syndrome of subacute onset (9 weeks to nadir). In the etiological study, high titers of GAD-ab were found, but these only recognized the GAD67 isoform and not the GAD65. Screening of GAD67-ab should be considered in late-onset cerebellar ataxia when GAD65-ab are absent.

1. Introduction

Autoantibodies against glutamic acid decarboxylase (GAD-ab) associate with several neurologic disorders such as stiff-person syndrome, cerebellar ataxia, and limbic encephalitis (Saiz et al., 2008). GAD-ab recognize the smaller isoform of the enzyme, GAD65, localized at the intracellular presynaptic site of inhibitory synapses, and high titers of GAD65-ab are currently a biomarker of these disorders. The presence of antibodies against the larger isoform, GAD67, is rarely included in clinical testing despite it has been reported in a high proportion of patients with GAD-ab associated disorders, usually accompanying GAD65-ab (Meinck et al., 2001). It is unknown whether GAD67-ab alone associate with a specific neurological syndrome. We report here the case of a patient who developed cerebellar ataxia along with autoantibodies restricted to the GAD67 isoform.

2. Case report

In November 2015, a 59-year-old woman presented with sudden onset of dizziness, vomiting and vertigo for 15 days. She denied sensory deficits and other focal neurologic or systemic symptoms, but complained of blurred vision. Neurological examination was remarkable for vertical nystagmus, horizontal diplopia, and mild right-arm dysmetria on finger-to-nose testing. Routine laboratory evaluation, brain MRI and MR-angiography were normal (Fig. 1). She was first treated for vertigo with betahistine and sulpiride, but during the ensuing 9 weeks she had a rapidly progressive neurological deterioration evolving to a pancerebellar syndrome. At examination, she had nystagmus on upward gaze, scanning speech, asymmetric dysmetria involving the 4 limbs, intention tremor, hypotonia, brisk deep tendon reflexes, right ankle clonus, and wide-based ataxic gait requiring bilateral assistance to walk. Cognition was normal. There were no other cranial nerve deficits, and she had normal strength. The score in ICARS scale was 61/100 (Trouillas et al., 1997).

Repeat brain MRI was normal (Fig. 1). Blood tests including blood count and renal function were normal. Specific studies for metabolic or nutritional deficits (copper, thyroid function, vitamins E and B12), autoimmune or inflammatory disorders (transglutaminase antibodies, antinuclear and Ro antibodies, angiotensin-converting enzyme) and tumor markers were unremarkable. Whole-body CT scan was normal. Two CSF studies were normal except for a CSF-restricted IgG oligoclonal band. Neuronal antibody studies using frozen rat brain tissue immunohistochemistry revealed a pattern of reactivity suggestive of GAD-ab in CSF and serum (Fig. 2). Immunoblot using 2 commercial kits containing GAD65 (Euroimmune and Ravo) were both negative in serum and CSF. Serum was also negative for GAD65 using a commercial RIA analysis. An

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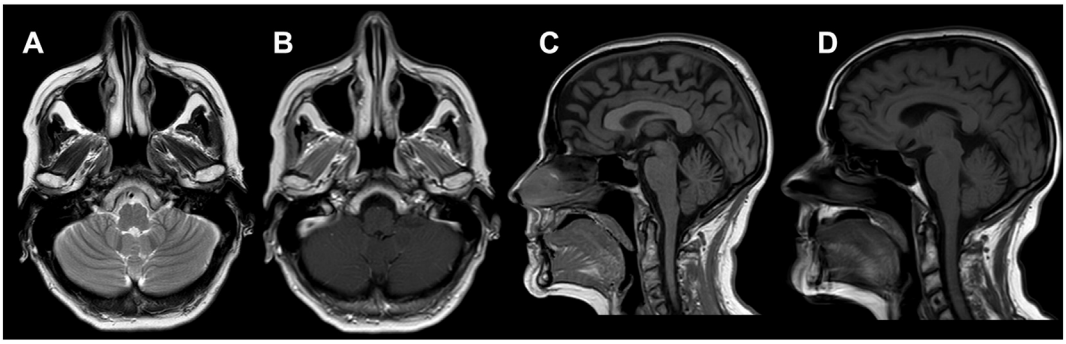

Fig. 1. Brain MRI. At diagnosis axial T2 (A) and axial T1-gadolinium (B) did not reveal structural abnormalities or contrast enhancement. In sagittal slices, the volume of the cerebellar vermis was stable in a control (D) at 10 months from clinical onset, 6 weeks after the first neuroimaging (C).

in house cell-based assay using HEK293 cells transfected with recombinant human GAD65 or GAD67 (Gresa-Arribas et al., 2015) showed that both serum and CSF contained antibodies restricted to the GAD67 isoform. The titer of GAD-abs was evaluated by serial dilutions in immunohistochemistry until the specific immunoreactivity disappeared. Serum titer was 1:3600 and CSF titer 1:1200. The index of GAD-ab was calculated as previously described (Saiz et al., 2008) and it was 74.8, indicating a strong intrathecal synthesis.

The patient was treated with intravenous metilprednisolone (1 g per day during 3 days) and 3 pulses of IVIg (2 g/kg distributed along 5 days), with only mild clinical improvement. Subsequently, she received rituximab (2 cycles of 1 g separated by 15 days) and 1 month later 1 g of cyclophosphamide (the first of 3 cycles in a monthly basis). The patient has not developed diabetes mellitus. In the last follow-up, 8 months after symptom onset, the patient was slightly better; the ICARS was 48/100 and she was able to walk a few steps without assistance.

3. Discussion and conclusions

To our knowledge, this is the first report of cerebellar ataxia associated with antibodies exclusively against GAD67. The clinical picture consistent with late-onset rapidly progressive cerebellar degeneration in a woman close to her sixties, is undistinguishable from the phenotype associated with GAD65-ab (Ariño et al., 2014; Honnorat et al., 2001). High titer of GAD-ab and the demonstration of intrathecal synthesis of antibodies are key findings for the diagnosis of GAD-ab associated neurological disorders (Jarius et al., 2010; Saiz et al., 1997). This case satisfies both conditions. Other cases of neurological syndrome associated exclusively with GAD67-ab include 2 patients with stiff-person syndrome who presented GAD immunoreactivity in serum revealed by immunohistochemistry, negative by immunoblot but recognizing native GAD67 (Johnstone and Nussey, 1994).

Although the significance of GAD67-ab is poorly understood, a recent study focused in the systematic determination of GAD-ab in a

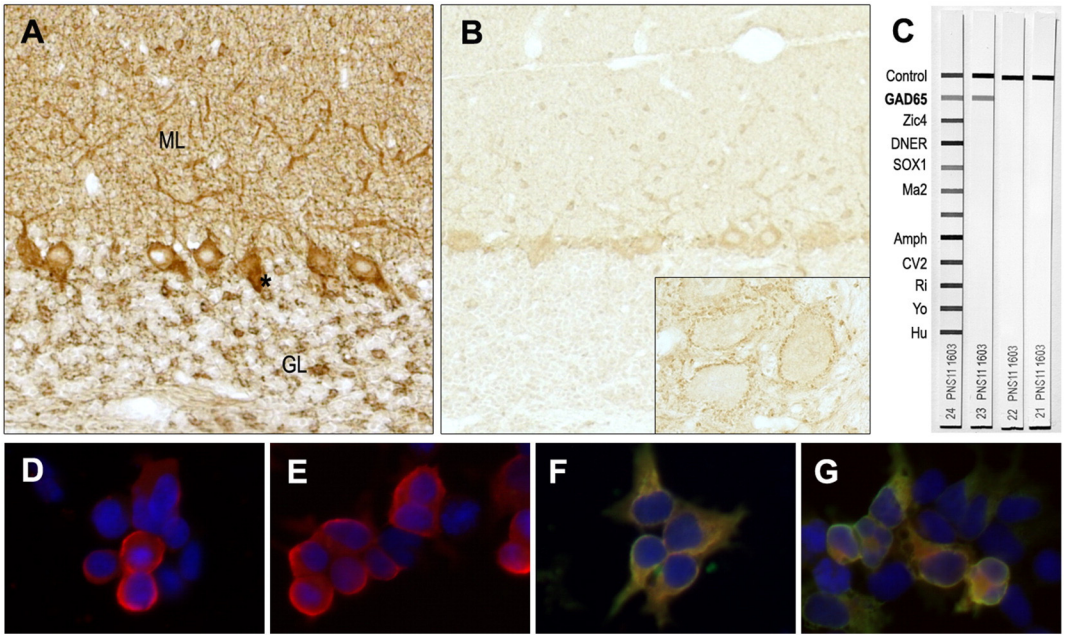

Fig. 2. Immunoassays for GAD-ab detection. (A, B) Immunohistochemistry on rat cerebellar tissue shows the characteristic staining of the presynaptic inhibitory terminals, especially the axon hillock of Purkinje cells (*), the granular layer (GL) and dendritic tree of Purkinje cells penetrating the molecular layer (ML). At regular conditions for antibody screening this pattern was stronger in the CSF (A, dilution 1:2) compared with the serum (B, dilution 1:500), which had the immunostaining more apparent essentially in the cerebellar deep nuclei (inset, ×40). (C) A commercial immunoblot was negative for GAD65-ab in serum and CSF (strips 22 and 21 respectively), in contrast to the serum from another patient with cerebellar ataxia and GAD-ab (strip 23). (D-E) Merged images of a cell-based assay using HEK293 cells transfected with GAD65 (D, E) or GAD67 (F, G) showing selective recognition of the GAD67 isoform. The yellow signal shows the merged immunofluorescence of the commercial antibody and the patient's antibody recognizing GAD67 in serum (F) and CSF (G). This contrasts to the GAD65 assay, which is only red due to the absence of reactivity in serum (D) and CSF (E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

large cohort of patients with neurological disorders showed that in patients with GAD65-ab, the study of CSF always showed the presence of GAD67-ab, despite 15% of paired sera were GAD67-ab negative, indicating an active antibody synthesis within the CNS (Gresa-Arribas et al., 2015).

The absence of GAD65-ab in our patient (and presumably in 2 previous patients with stiff-person syndrome) argues against the presence of GAD67-ab being the result of cross-reactivity or an epitope-spreading phenomenon secondary to a dominant epitope in GAD65. Moreover, it raises the question whether GAD65-ab are the most specific biomarker for neurological disorders or, in contrast, we should consider the routine screening of GAD67-ab or both in patients with suspected GAD-ab associated syndromes. It is unknown how antibodies against GAD65 or GAD67 reach their intracellular antigens, so the same concerns about the pathogenic role of GAD65-ab are applicable to GAD67-ab (Alexopoulos and Dalakas, 2010). Considering only their diagnostic value, this case illustrates the importance of GAD67-ab as autoimmune biomarker to identify patients that may respond to immunotherapy and the value of the antibody analysis by immunohistochemistry that detect antibodies against both GAD isoforms in contrast to the most common routine techniques (radioimmunoassay and immunoblot). The therapeutic strategy in patients with cerebellar ataxia and GAD-ab is not clear and the response is variable, but an early diagnosis is mandatory to increase the likelihood of a better outcome (Ariño et al., 2014). In summary, this study shows that patients with neurological symptoms suggestive of GAD-autoimmunity, but GAD65-ab negative, may have antibodies against GAD67.

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Resumen en Castellano

Introducción y objetivos

Esta tesis se centra en síndromes neurológicos asociados a anticuerpos contra el enzima descarboxilasa del ácido glutámico (anti-GAD). Estos anticuerpos se han descrito asociados a multitud de síndromes neurológicos, enfermedades endocrinológicas, e incluso se encuentran en una pequeña proporción de sujetos sanos. El rol de estos anticuerpos en cada una de estas entidades no está perfectamente establecido y, desde el punto de vista de la neurología clínica, es especialmente relevante conocer qué implicaciones clínicas tiene el hallazgo de estos anticuerpos en pacientes con determinados síndromes neurológicos.

La presencia de anticuerpos dirigidos contra moléculas, antígenos, del propio individuo (autoanticuerpos) se ha asociado a enfermedades autoinmunes del sistema nervioso, pero con diferente rol. Si bien en unos casos estos autoanticuerpos son directamente patogénicos (enfermedades mediadas por anticuerpos), en otros son marcadores subrogados de un proceso autoinmune subyacente, pero no los son responsables de la inmunopatogénesis de la enfermedad (enfermedades asociadas a anticuerpos). En este último caso, la detección de anticuerpos es la base de tests específicos cuyo valor más importante es ser biomarcador diagnóstico. De los trabajados derivados de Witebsky se establecieron los criterios para determinar la patogenicidad de un determinado autoanticuerpo, que incluyen la demostración directa de anticuerpos circulantes en pacientes dirigidos contra un antígeno específico, la capacidad de inducir la producción de anticuerpos en animales de experimentación tras inmunización activa con dicho antígeno y por último,

la inducción de cambios patológicos en dichos modelos experimentales (tras inmunización activa o por transferencia directa de anticuerpos de pacientes) que remeden la enfermedad en humanos. El paradigma de enfermedad inmunomediada afectando al sistema nervioso central es la encefalitis NMDAR, mediada por anticuerpos contra el receptor glutamatérgico N-metil-D-aspartato de la membrana neuronal. Ejemplos de enfermedad asociada a, pero no inmunomediada por, anticuerpos son gran parte de los síndromes neurológicos paraneoplásicos, donde la expresión ectópica de antígenos neurales por un tumor da lugar a una respuesta autoinmune generalmente mediada por citotoxicidad. Los anticuerpos detectados en estos casos, ayudan a diagnosticar pacientes con un síndrome neurológico de origen paraneoplásico y a la búsqueda precoz de un tumor oculto. Pero además, este distinto papel patogénico con frecuencia se asocia a distinta respuesta a la inmunoterapia; pues aquellos mediados por anticuerpos responden de forma más contundente al tratamiento que logra reducir efectivamente el nivel de anticuerpos.

En el caso de los anticuerpos anti-GAD la evidencia respecto a su patogenicidad es controvertida. Estudios *in vitro* sugieren que pueden tener un papel patogénico, pero no se ha demostrado que los anticuerpos puedan alcanzar su antígeno intracelular y estudios experimentales *in vivo* no logran reproducir robustamente ningún aspecto clave de la enfermedad. Así pues, si no son patogénicos, ¿qué valor tiene su detección? ¿Son exclusivamente biomarcadores de determinados síndromes neurológicos autoinmunes? ¿Pueden ayudar a predecir el fenotipo clínico, la respuesta a la inmunoterapia o la presencia de un tumor oculto?

La descarboxilasa del ácido glutámico (GAD) es la enzima limitante de la síntesis del principal neurotransmisor inhibidor del sistema nervioso central, el ácido γ–aminobutírico (GABA). Este enzima se expresa selectivamente en neuronas GABAérgicas y células pancreáticas β. El enzima tiene dos isoformas, derivadas de dos genes diferentes, GAD65 (una forma asociada a la membrana de la vesícula sináptica o microvesículas pancreáticas) y GAD67 (forma soluble encargada de la producción basal de GABA, detectable únicamente en el sistema nervioso central). Comparten un 65% de identidad y difieren principalmente en la región amino-terminal.

El GAD es el principal autoantígeno presente en diabetes mellitus tipo 1 (DM1) y los anticuerpos contra anti-GAD se detectan hasta en un 80% de los pacientes con DM1 de reciente diagnóstico. Se encuentran títulos altos de anti-GAD, del orden de las 100 veces respecto a los títulos de DM1, en pacientes con síndrome de persona rígida (SPR), en pacientes con ataxia cerebelosa de inicio tardío asociada a DM1 o a síndrome poliglandular autoinmune, pacientes con epilepsia o encefalitis límbica y en otros grupos de pacientes menos frecuentes como pacientes con nistagmus vertical, temblor palatino y disfunción troncoencefálica.

Los anti-GAD pueden ser detectados a muy bajas concentraciones mediante inmunoensayo (ELISA o RIA). Se desconoce el valor diagnóstico de títulos bajos de anti-GAD65 en suero, que pueden estar presentes en población sana (1%) y en otros síndromes neurológicos (5%).(Meinck et al., 2001).

El SPR es un trastorno del sistema nervioso central caracterizado por rigidez muscular progresiva de predomino en la musculatura del tronco con

espasmos, secundaria a la co-contracción de músculos agonistas y antagonistas causada por la descarga involuntaria de unidades motoras en reposo. En el 80% de los pacientes con SPR o sus variantes (SLS-*stiff limb syndrome*, PERM-*Progressive encephalomyelitis with rigidity and myoclonus*) se encuentran anti-GAD a títulos altos, tanto en suero como en líquido cefalorraquídeo, por lo que son un excelente marcador de la enfermedad aunque no se requieren para diagnóstico. El síndrome se asocia frecuentemente a otras enfermedades autoinmunes organoespecíficas, especialmente a DM1 (30-60%), con una relación temporal variable. Por ser el primer trastorno neurológico asociado a la inmunidad anti-GAD65 y el más prevalente, la mayoría de los trabajos enfocados a estudiar la fisiopatología han sido hechos en este grupo de pacientes en comparación a pacientes diabéticos.

La ataxia cerebelosa, el segundo trastorno neurológico más frecuentemente asociado a anti-GAD, consiste en un síndrome cerebeloso de curso insidioso e inicio en la edad adulta en que el síntoma prínceps es la ataxia de la marcha, aunque puede acompañarse de dismetría, nistagmo o disartria. Parece compartir las mismas características epidemiológicas, clínicas e inmunológicas que el SPR idiopático. En aquellos pacientes con mayor índice de síntesis intratecal, puede coexistir ataxia cerebelosa con SPR. En estos pacientes la inmunoterapia parece mejorar fundamentalmente el SPR pero no el síndrome cerebeloso. No obstante, no se conoce el pronóstico a largo plazo de estos pacientes.

La prevalencia de títulos elevados de anti-GAD en epilepsia es mayor de la que series iniciales sugerían si se selecciona la población a estudio. En epilepsia del lóbulo temporal (ELT) de inicio en edad adulta llega al 15% (9-

17%, según la serie consultada). Hay un subgrupo de pacientes en los que se ha descrito el inicio agudo en forma de encefalitis límbica (con crisis temporales y criterios radiológicos), que evolucionan a epilepsia temporal farmacorresistente pese al tratamiento inmunosupresor o inmunomodulador. Se observa una disminución de los títulos de anticuerpos tras inmunoterapia en estos pacientes con encefalitis límbica, pero no desaparece la síntesis intratecal ni quedan libres de crisis. En un pequeño porcentaje puede coexistir ELT con SPR.

Una cuestión fundamental es qué determina el variado fenotipo clínico de los trastornos neurológicos contra el mismo autoantígeno. No parece existir una relación entre el título de anti-GAD y la presentación clínica. Ni siquiera es suficiente para justificar el desarrollo de un síndrome neurológico en pacientes con DM1 y títulos elevados en seguimientos clínicos prolongados. Estudios in vitro han demostrado la capacidad de los anti-GAD de inhibir la síntesis de GABA cuando proceden de pacientes con SPR pero no con DM1. Todo ello sugiere una diferente especificidad de epítopos de GAD. Esta diferencia se demostró en estudios realizados fundamentalmente en la década de los 90. Existen epítopos conformacionales cuya presencia no se puede detectar por Western Blot pero sí mediante otras técnicas. En general se considera que los anticuerpos de pacientes con DM1 reconocen epítopos conformacionales de GAD65, mientras que los sueros de pacientes con SPR reconocen principalmente epítopos lineales en la región aminoterminal de la molécula GAD65. Pero además coexiste una reactividad diferente a la isoforma GAD67 y estudios previos demuestran reactividad a epítopos conformacionales de GAD67 en un 50-60% de los casos de pacientes con SPR seropositivos para GAD65 (aunque a títulos más bajos), mientras en

DM1 esta reactividad no llega a un 12% y se atribuye a un fenómeno de reacción cruzada. Se desconoce la frecuencia de anti-GAD67 en pacientes con trastornos neurológicos diferentes a SPR.

A pesar de los esfuerzos realizados hasta la fecha, se desconoce el significado de estos autoanticuerpos y se cuestiona su rol patogénico. El problema fundamental es que se trata de una proteína citosólica. Los autoantígenos intracelulares se consideran en general marcadores subrogados de inmunidad celular. En la búsqueda de otros anticuerpos más relevantes se ha descrito anticuerpos contra GABARAP (proteína asociada al receptor de $GABA_A$) en un 70% de pacientes con SPR y títulos elevados anti-GAD65. Pero al igual que GAD, se trata de una proteína intracelular, por lo que su relevancia es cuestionable. En relación a antígenos de superficie neuronal adicionales que pudiesen ser más relevantes, se han identificado 2 anticuerpos en un limitado número de pacientes. Por una parte, nuestro grupo ha descrito una pequeña de serie de pacientes con títulos elevados de anti-GAD y anticuerpos contra el receptor de $GABA_B$. Se trata de 2 casos de encefalitis y 1 caso de ataxia cerebelosa, todos paraneoplásicos. En otra publicación reciente se ha descrito una serie de casos del espectro clínico del SPR con anti-GAD que presentan anticuerpos contra la subunidad 1 del receptor de glicina (neurotransmisor con efecto inhibidor sináptico a nivel medular y troncoencefálico). En estos escasos pacientes, la respuesta al tratamiento inmunosupresor parece ser más constante. No se ha investigado de forma sistemática la presencia de anticuerpos contra estos nuevos autoantígenos potencialmente implicados en la disfunción gabaérgica que comparten estos trastornos neurológicos asociados a inmunidad anti-GAD, ni qué implicaciones clínicas tiene la

detección de estos anticuerpos en el contexto de determinados síndromes neurológicos.

Pese a que se ha demostrado la expresión de GAD en tejido tumoral de pacientes con un síndrome neurológico y niveles elevados de anti-GAD, los casos asociados a neoplasia son sustancialmente menos frecuentes que los que no. Por este motivo, los anticuerpos anti-GAD no pueden ser incluídos en el grupo de anticuerpos onconeuronales bien caracterizados, que llevan a un diagnóstico definitivo de síndrome neurológico paraneoplásico. El pronóstico, sin embargo, es bien diferente. Actualmente no es posible distinguir los casos paraneoplásicos en el momento del diagnóstico de un síndrome neurológico asociado a inmunidad anti-GAD.

Con estos antecedentes, esta tesis está enfocada a investigar los determinantes inmunológicos de la diversidad fenotípica y el rol de los anticuerpos anti-GAD como biomarcador pronóstico o predictivo de respuesta al tratamiento o a la presencia de cáncer. Por objetivos:

1. Investigar las diferencias en la respuesta inmune entre diferentes fenotipos clínicos asociados a anti-GAD (síndrome de la persona rígida, ataxia cerebelosa, epilepsia y encefalitis límbica)

2. Investigar el pronóstico a largo plazo y el efecto de la inmunoterapia en pacientes con ataxia cerebelosa

3. Explorar factores predictivos de neoplasia en pacientes con síndromes neurológicos y anticuerpos anti-GAD

4. Determinar el valor pronóstico de los anticuerpos anti-GAD en pacientes con síndromes del espectro de la persona rígida

Resultados

Para cada uno de los objetivos específicos, se ha realizado un trabajo con publicación independiente. A continuación se resume la metodología y los hallazgos más importantes de cada uno de ellos.

OBJETIVO 1

Antibodies to inhibitory synaptic proteins in neurological syndromes associated with glutamic acid decarboxylase autoimmunity. PLoS One. 2015 Mar 16;10(3):e0121364. doi: 10.1371/journal.pone.0121364.

En este artículo se estudió el suero y líquido cefalorraquídeo (LCR) de 106 pacientes con síndromes neurológicos asociados a anticuerpos anti-GAD sin neoplasia asociada: 39 pacientes con ataxia cerebelosa, 32 con síndrome de la persona rígida (SPR), 18 con epilepsia y 17 con encefalitis límbica. El título de GAD65 se cuantificó mediante ELISA. Se determinó si los epítopos reconocidos por los anticuerpos anti-GAD en GAD65 y GAD67 eran lineales con inmunoblot. Mediante ensayo celular usando células HEK293 expresando alguno de los 3 dominios funcionales del GAD65 (N-terminal, dominio central con la región catalítica o el C-terminal) se investigaron los dominios inmunodominantes. Se determinaron anticuerpos adicionales contra GAD67, receptor de $GABA_A$, de glicina, GABARAP y gefirina usando un ensayo celular con HEK293 transfectadas con el antígeno correspondiente. Se investigó la internalización de anti-GAD en cultivos de neuronas hipocampales de rata.

El título de anticuerpos contra GAD65 en LCR fue más elevado en pacientes con encefalitis límbica y ataxia cerebelosa comparado con el de los pacientes con SPR ($p = 0,02$). Se identificó anti-GAD67 en el 81% de los

sueros y 100% del LCR de todos los pacientes. Los anticuerpos reconocieron epítopos lineales de GAD65 en el 98% de los pacientes y de GAD67 en el 42% ($p < 0,001$). Se identificó reactividad contra el dominio central catalítico de GAD65 en el 93% de los sueros y contra los 3 dominios en el 22% de los sueros pero el 74% de los LCR ($p < 0,001$). En 6 pacientes se demostraron anticuerpos contra el receptor $GABA_A$ y en otros 6 contra el receptor de glicina sin asociarse a ninguna distinción clínica. En ninguno de los pacientes se encontraron anticuerpos contra glicina o GABARAP. Finalmente, los anticuerpos anti-GAD no fueron internalizados por neuronas hipocampales viables. Estos hallazgos muestran que, independientemente del síndrome neurológico, la respuesta inmune anti-GAD en el LCR es más extensa que la encontrada en el suero. Además, no en este estudio no se encontró ninguna asociación específica entre el fenotipo clínico y la presencia de anticuerpos contra otras proteínas de la sinapsis inhibitoria.

OBJETIVO 2

Cerebellar ataxia and glutamic acid decarboxylase antibodies: immunologic profile and long-term effect of immunotherapy. JAMA Neurol. 2014 Aug;71(8):1009-16. doi:10.1001/jamaneurol.2014.1011.

Este trabajo incluyó una cohorte de estudio de 34 pacientes con ataxia cerebelosa y anticuerpos anti-GAD. Se realizó un estudio retrospectivo de esta cohorte seleccionada entre aquellos pacientes con inmunorreactividad contra GAD identificados en nuestro centro de referencia de trastornos neurológicos autoinmunes en un periodo de 9 años. Se estudió el perfil inmunológico (prevalencia de GAD67 y anticuerpos adicionales contra autoantígenos de la sinapsis inhibitoria) con una metodología análoga al

trabajo anterior. Se analizaron las características clínico-inmunológicas y se buscaron los factores predictivos de respuesta a la inmunoterapia en un subgrupo de 25 pacientes con información a largo plazo (mediana: 5,4 años; rango intercuartílico: 3,1 – 10,3 años) mediante un análisis estadístico multivariable.

La edad media de los pacientes fue 58 años (rango: 33-80); 28 (82%) eran mujeres. Nueve pacientes (26%) padecieron episodios de disfunción cerebelosa, troncoencefálica transitoria o de vértigo fluctuante varios meses antes de desarrollar el cuadro cerebeloso. La presentación clínica fue subaguda, desarrollándose en un periodo de semanas en 13 pacientes (38%). Nueve pacientes (26%) presentaron síntomas del espectro del SPR simultáneamente. En 29 pacientes (85%) se encontraron trastornos autoinmunes organoespecíficos (diabetes mellitus tipo 1, tiroiditis y otros). Veinte de los 25 pacientes con seguimiento recibió inmunoterapia (inmunoglobulinas endovenosas en 10 y esteroides combinados con inmunoglobulinas o algún inmunosupresor en otros 10); 7 delos cuales (35%) mejoró. Los factores de respuesta clínica encontrados fueron 2: el inicio subagudo (OR 0,5; IC95% 0,25 – 0,99; p = 0,047) y el inicio precoz de la inmunoterapia (OR 0,98; IC95% 0,96-0,99; p = 0,01). La frecuencia de anti-GAD67 encontrada fue 71% en suero y 100% en LCR, similar a la prevalencia de estos anticuerpos en un grupo control de pacientes con SPR. Se encontraron anticuerpos contra el receptor de glicina en 4 pacientes, que no se correlacionó con el pronóstico ni otra característica clínica específica.

OBJETIVO 3

Paraneoplastic Neurological Syndromes and Glutamic Acid Decarboxylase Antibodies. JAMA Neurol. 2015 Aug;72(8):874-81. doi: 10.1001/jamaneurol.2015.0749.

Este trabajo es un estudio retrospectivo de una serie de pacientes con anti-GAD, cáncer, un síndrome neurológico con criterio temporal de paraneoplásico (Graus et al., 2004) y ausencia de otros anticuerpos bien caracterizados de síndromes paraneoplásicos. Se recogieron 15 pacientes con estas características identificados entre 1995 y 2013 en nuestro centro de referencia de síndromes paraneoplásicos y se investigaron las características clínicas e inmunológicas. Además se realizó una revisión de la literatura de 19 casos publicados con adecuada información. Se comparó la serie propia con los 106 casos sin criterios de paraneoplásico (no-PN) recogidos en el primer trabajo. Mediante regresión logística se buscaron factores predictivos de cáncer.

De los 15 pacientes con cáncer, 8 desarrollaron un síndrome neurológico paraneoplásico clásico (5 encefalitis límbica; 1 encefalomielitis paraneoplásica; 1 degeneración cerebelosa paraneoplásica y 1 síndrome de opsoclono-mioclono). En comparación a los 106 casos no-PN, los pacientes con síndromes paraneoplásicos eran mayores (edad mediana de 60 vs. 48 años, $p = 0,03$), más frecuentemente hombres (60% vs. 13%, $p < 0,001$) y mayor prevalencia de anticuerpos adicionales contra receptores de superficie neuronal (53% vs. 11%, $p < 0,001$), fundamentalmente contra el receptor $GABA_B$. Los tumores más frecuentemente involucrados eran de pulmón (6) y de origen tímico (4). El riesgo de cáncer subyacente fue mayor en aquellos casos que se presentaban con un síndrome diferente al SPR o

ataxia cerebelosa (OR 10,5; IC95% 3,2 – 34,5), fundamentalmente en forma de síndrome neurológico paraneoplásico clásico, o en casos con anticuerpos adicionales contra receptores de superficie neuronal (OR 6,8; IC95% 1,1-40,5). Los 19 casos previamente publicados, en comparación a nuestra serie, comprendían una mayor frecuencia de SPR (74% vs. 13%, p= 0,001) y tenían una mayor tasa de respuesta al tratamiento (79% vs. 27%, p = 0,005). Teniendo en cuenta los 34 pacientes (la serie propia y los previamente publicados), la presentación como SPR y la presencia de un tumor tímico son predictores de mejoría clínica.

OBJETIVO 4

Clinical and Immunologic Investigations in Patients With Stiff-Person Spectrum Disorder. JAMA Neurol. 2016 Jun 1;73(6):714-20. doi:10.1001/jamaneurol.2016.0133.

Este trabajo, también un estudio retrospectivo, incluye una cohorte de 121 pacientes seleccionados con criterios clínicos, con síndromes del espectro de la persona rígida. Se investigó el perfil inmunológico (presencia de anticuerpos contra GAD, receptores de $GABA_A$, $GABA_B$, glicina, transportadores de glicina 1 y 2, gefirina y DPPX), la asociación clínico-inmunológica y el valor pronóstico en un subgrupo de 75 pacientes con un seguimiento de 18 meses de mediana.

La edad media de los pacientes fue de 51 años (rango intercuartílico 40-61 años) y 75 (62%) eran mujeres. Cincuenta pacientes (41,3%) tenían SPR, 37 (30,6%) SPR-plus (con criterios de PERM o síntomas parciales), 24 (19,8%) tenían un síndrome de miembro rígido (*stiff-limb*), y 10 (8,3%) SPR o stiff-limb unido a otro síndrome neurológico asociado a inmunidad anti-

GAD (ataxia cerebelosa, epilepsia o encefalitis). Anti-GAD se encontraron en 52 pacientes (43%, 2 con anticuerpos adicionales contra receptor $GABA_A$), 24 (19,8%) anticuerpos contra receptor de glicina (2 con anti-GAD), 5 (4,1%) presentaron otros anticuerpos, y 40 (33,1%) resultaron negativos para cualquiera de los anticuerpos testados. En ningún caso se identificaron anticuerpos contra el transportador de glicina o contra gefirina. Entre los principales grupos inmunológicos (anti-GAD, anticuerpos contra el receptor de glicina y seronegativos), aquellos pacientes con anti-GAD eran con mayor frecuencia mujeres (45 [86,5%] de 52; 8 [36,4%] de 22; y 18 [45 %] de 40 respectivamente; p<0,001), presentaban mayor frecuencia de trastornos autoinmunes sistémicos (34 [65,4%] de 52; 7 [31,8%] de 22; y 13 [32,5%] de 40 respectivamente; p=0,004), y mayor retraso en ser testados para presencia de anticuerpos (mediana de 3 vs 0,5 y 1 año; p< 0,001). Los pacientes con anti-GAD presentaban con mayor frecuencia SPR (27 [51,9%] de 52) o síndromes de solapamiento (8 [15,4%] de 52) que aquellos pacientes con anti-receptor de glicina (5 [22,7%] y 0 de 22 respectivamente), los cuales desarrollaban a menudo un SPR-plus (12 [54,5%] de 22 vs. 7 [13,5%] anti-GAD). En el estudio multivariable, la gravedad clínica (p=0,001) y el grupo inmunológico (p=0,01) se asociaban de forma independiente con el pronóstico (medido como la combinación de mejoría clínica y un estado funcional ambulatorio en la última visita). En comparación a los pacientes con anti-receptor de glicina, aquellos con anti-GAD tuvieron un peor pronóstico (OR 11,1; IC95% 2,3-53,7; p=0,003). Los pacientes seronegativos, sin embargo, tuvieron un pronóstico similar a los pacientes con anti-receptor de glicina (OR 4,2; IC95% 0,9 – 20; p = 0,07)

Discusión

Esta tesis incluye la cohorte más grande de pacientes con síndromes neurológicos y anticuerpos anti-GAD. Se ha realizado un estudio exhaustivo de la respuesta inmune asociada, incluyendo la reactividad contra epítopos inmunodominantes y la presencia de anticuerpos adicionales, tanto contra la isoforma GAD67 como contra antígenos de superficie neuronal, mostrando que esta respuesta inmune es relativamente homogénea entre los diferentes síndromes neurológicos con la metodología empleada, y no se han detectado asociaciones clínico-inmunológicas particulares. Es relevante, no obstante, que las características de esta respuesta difieren entre el suero y LCR, y es más amplia en este último compartimento, sugiriendo una maduración específica a este nivel. Sea cual sea el mecanismo de esta maduración, este hallazgo tiene implicaciones a la hora de investigar el rol de estos anticuerpos en síndromes neurológicos donde puede resultar esencial usar muestras de LCR en lugar de suero.

Los trabajos centrados en los objetivos 2-4 muestran el valor predictivo o pronóstico de estos anticuerpos más allá de indicar un origen autoinmune del síndrome neurológico. En los pacientes con ataxia cerebelosa, que pueden desarrollar depleción neuronal selectiva de células de Purkinje según los escasos estudios necrópsicos, los hallazgos de nuestro estudio muestran que se benefician del tratamiento inmunomodulador y pueden mantener un buen pronóstico funcional a largo plazo. Uno de los factores de respuesta al tratamiento en nuestro estudio es el tiempo desde el diagnóstico al inicio del tratamiento, teniendo mayor posibilidad de respuesta cuanto más temprano se inicia la inmunoterapia. Existe, pues, una ventana terapéutica donde la disfunción

es potencialmente reversible y, aunque los pacientes con inicio subagudo parece que son los que responden con mayor frecuencia, el ensayo terapéutico con inmunoterapia debería intentarse en todos los pacientes con ataxia cerebelosa y anti-GAD. Para identificar precozmente estos pacientes, se incide en las características clínicas que deben hacer sospechar inmunidad anti-GAD asociada a un síndrome cerebeloso. En ese sentido, es relevante la frecuencia de episodios transitorios de disfunción neurológica en los meses previos a desarrollar el cuadro cerebeloso, o la frecuencia de síntomas del espectro del síndrome de la persona rígida que coexisten en estos pacientes.

Aunque la inmunidad anti-GAD raramente se asocia a la presencia de una neoplasia oculta (prevalencia inferior al 15% entre pacientes con anti-GAD en un centro de referencia para trastornos neurológicos paraneoplásicos), el estudio realizado en esta cohorte halla factores predictivos que deben elevar la sospecha de cáncer en pacientes con síndromes neurológicos. El tipo de síndrome es el factor más robusto, y los pacientes que se presentan con síndromes neurológicos atípicos en el contexto de inmunidad anti-GAD (diferentes a SPR o ataxia cerebelosa) deben someterse al despistaje de una neoplasia oculta. Además, el riesgo se incrementa en los pacientes varones, de edad avanzada o en presencia de anticuerpos adicionales contra superficie neuronal.

En el último trabajo se demuestra que los síndromes del espectro de la persona rígida, definidos clínicamente, son heterogéneos desde un punto de vista inmunológico y se asocian a gran número de anticuerpos además de anti-GAD, aunque este sigue siendo el grupo más frecuente. Otros grupos frecuentes son pacientes con anticuerpos contra el receptor de

glicina y pacientes sin anticuerpos. La distribución relativa de estos grupos debe interpretarse con precaución, pues probablemente existe un importante sesgo de selección por el tipo de pacientes cuyas muestras son referidas a un centro especialista. Por el contrario, son relevantes las asociaciones clínico-inmunológicas obtenidas en nuestro estudio, no tan vulnerables a este sesgo. Encontramos que cualquiera de los síndromes puede aparecer entre los 3 principales grupos inmunológicos hallados y es la clasificación inmunológica, pero no el tipo de síndrome, el factor que se relaciona de forma independiente con el pronóstico. Los pacientes con anti-receptor de glicina son los que mejor pronóstico tienen a pesar de una presentación más grave, lo que puede explicarse por la mayor respuesta a la inmunoterapia que presentan los pacientes con anti-receptor de glicina respecto a los pacientes con anti-GAD. Los anticuerpos no son actualmente un criterio diagnóstico. A la luz de estos hallazgos, no deben serlo; existen pacientes que, con las técnicas de laboratorio usadas actualmente, son definitivamente seronegativos. Sin embargo, dadas las implicaciones pronósticas, está justificado incluir el despistaje de los principales grupos inmunológicos en la valoración inicial de pacientes con síndromes del espectro de la persona rígida.

Los resultados de esta tesis contribuyen a clarificar qué valor tiene el hallazgo de anticuerpos anti-GAD en pacientes con determinados síndromes neurológicos y a caracterizar el perfil de algunos de los grupos clínicos asociados a anti-GAD más desconocidos, como son pacientes con ataxia cerebelosa o síndromes paraneoplásicos.

Conclusiones

1. La presencia adicional de anticuerpos contra antígenos de la sinapsis inhibitoria, una reactividad diferente contra alguna de las isoformas de GAD o contra algún epítopo particular de GAD65, no determinan la diversidad fenotípica en síndromes neurológicos asociados a anticuerpos anti-GAD

2. La respuesta inmune contra GAD difiere en suero y líquido cefalorraquídeo, sugiriendo un proceso de maduración intratecal en pacientes con síndromes neurológicos no paraneoplásicos

3. Los pacientes con ataxia cerebelosa y anticuerpos anti-GAD pueden responder a la inmunoterapia y mantener un buen estado funcional a largo plazo. La instauración del tratamiento de forma precoz, posiblemente ofrece una mayor probabilidad de respuesta

4. En el contexto de la inmunidad anti-GAD, los síndromes neurológicos paraneoplásicos tienen una presentación clínica y un perfil inmunológico diferente. Aquellos pacientes con síndromes neurológicos diferentes al síndrome de la persona rígida o ataxia cerebelosa deberían ser sometidos al despistaje de una neoplasia oculta

5. En pacientes con síndromes del espectro de la persona rígida, la clasificación inmunológica es un factor pronóstico independiente. Aquellos pacientes con anticuerpos anti-GAD tienen un peor pronóstico que aquellos seronegativos o que los pacientes con anticuerpos contra el receptor de glicina.

Epilogue

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