EXPLORING THE CLINICAL SPECTRUM OF GLUTAMIC ACID DECARBOXYLASE ANTIBODIES IN NEUROLOGICAL PATIENTS, THROUGH THE VALIDATION OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

Glutamic Acid Decarboxylase (GAD) antibodies are markers of type 1 diabetes mellitus (T1DM) and autoimmune neurological syndromes. The main neurological syndromes linked to GAD abs are stiff-person syndrome (SPS), cerebellar ataxia, limbic encephalitis and epilepsy. Titers of GAD abs in neurological patients are much higher than in diabetes. The meaning of low titres in neurological syndromes is still uncertain and the cut off for the relevance of GAD abs is debated. Moreover, commercial kits for detection of GAD abs are usually validated only in patients with T1DM.

Our work aims:
- to perform a clinical validation of a commercial ELISA assay for GAD abs detection in serum and cerebrospinal fluid (CSF) of patients with suspected neurological autoimmune diseases;
- to set up a clinically relevant cut-off for serum GAD abs;
- to explore the meaning of serum low-titre GAD abs in neurological patients;
- to investigate the presence of coexisting antineuronal antibodies in GAD abs seropositive patients;

We tested 330 patients with suspected autoimmune neurological syndromes for GAD abs by a modified commercial ELISA kit. Sera and CSF were titrated by multiple dilutions and the final values extrapolated by a mathematical model. When paired serum and CSF were available, intrathecal synthesis was calculated. Specimens were also tested for other antineuronal abs (onconeural and surface antigens) by a cell-based assay and immunoblot; GAD abs were searched also by immunoistochemistry on frozen rat brain and immunoblot.

Clinical features were reviewed by one observer blinded for GAD titers; he noted if a typical GAD-related autoimmune neurological syndrome was present; then a ROC curve was generated to find the best cut off that distinguishes clinically relevant from clinically not relevant titres.

Forty-three patients tested positive by optimized ELISA (serum GAD abs, range 6 – 2019681 UI/ml), 35 with available clinical information were included (14 males and 21 females, age range 15-82 years old). In 26/35 patients (74%) the final diagnosis belonged to the already reported spectrum of GAD-abs related diseases, specifically 10 patients diagnosed with encephalitis, 9 with SPS, 5 with ataxia and 2 with epilepsy. Among these, 12/26 failed to fit the criteria described in the methods and were noted as “Atypical”. 14 patients showed typical features. ROC analysis identified a cut off at 13000 IU/ml. Intrathecal synthesis of GAD abs were detected in 9 patients with serum high-titre GAD abs. One patient with a high GAD- abs titre showed co-reactivity with anti-Amphyphisin abs; 4/36 (11%) with low-titre GAD abs had a relevant neuronal surface antibodies positivity for anti-LGI1 (3) and anti-GlyR antibodies (1).

We confirmed clinical spectrum of GAD autoimmunity. The study validates a modified ELISA in routine detection of GAD abs in neurological patients, setting the threshold of 13000 IU/ml as a discriminant serum cut off to identify clinically relevant GAD abs.
INTRODUCTION

Glutamic Acid Decarboxylase

Glutamic Acid Decarboxylase (GAD) is an essential enzyme producing Gamma-aminobutyric acid (GABA) from Glutamate. GABA is the most important inhibitory neurotransmitter of the central nervous system (CNS) and is involved in the control of neurogenesis and tissue development. [1]

In mammals two isoforms of GAD exist, termed GAD67 and GAD 65 according to their molecular weights; they are coded by GAD1 and GAD2 genes and are located on chromosome 2 and 10, respectively. [2] Gene knockout studies underline the function of the isoforms: GAD1-/- mice have reduced GABA levels and die at the birth of the cleft palate [3]; GAD2-/- mice have normal basal GABA levels and develop fatal epilepsy and anxiety phenotype [4]. GAD67 is cytosolic and constantly active to produce basal levels of GABA. GAD65 is associated with the membrane of synaptic vesicles anchored to the cytoplasm-facing side and produces GABA when additional neurotransmitter is required (for example in stress responses). [5]

Both isoforms consist of three functional domains: an amino (N)-terminal domain, containing the membrane-binding sequence and anchoring GAD65 to vesicles, a middle PLP-binding domain containing the catalytic site of the enzyme and a carboxy domain (C-terminal) [6]. GAD is located mainly in neurons, but also in pancreatic ß-cells.[7]

GAD antibodies

In 1988 antibodies to GAD (GAD abs) were first described in a patient affected with stiff person syndrome (SPS), epilepsy and type-1 diabetes mellitus (T1DM). [8]. Two years later, GAD abs were described in patients affected by autoimmune diabetes. [9] GAD abs have subsequently also been reported in Batten disease, [10] type 1 autoimmune polyendocrine syndromes and further neurological syndromes like cerebellar ataxia, limbic encephalitis and palatal myoclonus [11].

Although SPS and T1DM are both associated with GAD abs, the diseases do not always develop in the same subject. Indeed, only 1/10000 diabetic patient is or will be affected with SPS; vice versa, only 30% of SPS patients are diabetic [12]. This fact is not due to structural differences between pancreatic and neuronal isoforms (they are coded by the same genes, although a post-translational modification cannot be excluded). While low accessibility of the immune system to GAD in the central nervous system and the low expression of Major Histocompatibility Complex (MHC) by neurons may explain the low incidence of SPS in diabetic patients, it does not explain the high proportion of SPS without diabetes (about 70%). Several data support a different pattern of immune response (humoral and cellular) in the two diseases although a common genetic background has been found (e.g. DQ1B*0201 is a susceptibility allele among SPS and T1DM patients). [13] As regards the autoantibody repertoire, patients with SPS have GAD abs binding conformational epitopes of middle domain, C-terminal and linear epitopes in the first 100 aminoacids of the N-terminal domain. [14] [15] [16]
Conformational blockage of the C-terminal epitope seems to block the activity of the enzyme and this fact does not occur in diabetic patients. [17] Also N-terminal GAD antibodies are usually not found in diabetic patients but their pathogenic role is uncertain. [18]

![Molecular conformation of GAD](image)

**Figure 1.** Molecular conformation of GAD (modified from [12]). Antibodies in SPS and T1DM are directed to different segments.

It has been well established that GAD abs in neurological diseases are higher than in diabetes. According to some authors, the pathogenic diversity depends on this difference rather than a different pattern of antigenic recognition of GAD. [19].

GAD65 abs are the principal biomarker due to greater autoantigenicity [20] but GAD67 antibodies are also found, especially in neurological patients. 50-60% of patients affected by a neurological syndrome whose serum harbours GAD abs also have anti-GAD67 abs, whereas only 12% T1MD have anti-GAD67 abs due to cross-reaction mechanisms. [21] [22] A recent paper by Gresa-Arribas et al. [16] showed that in a cohort of patients with GAD-correlated neurological diseases GAD67 abs were always found in the CSF, even in those cases whose serum was positive for GAD65 only.

The predominant IgG isotype was found to be IgG1; in a minority of patients IgG2 and IgG3 were usually found at lower titres. [23] [24]

Oligoclonal bands (OCB) are a frequent finding in CSF from patients affected by GAD abs diseases. Differences have been shown in the epitope specificity of GAD abs from serum and CSF from the same patient [25] suggesting intrathecal production by active B cells in the CNS.

**Pathogenicity of GAD antibodies**

The pathogenetic role of GAD abs in neurological diseases has not been established. The localization of the protein makes pathogenicity unlikely.
The heterogeneity of manifestations supports the hypothesis that other immune or cellular mechanisms are involved in the pathogenesis of the diseases. It has been reported that passive transfer from an SPS mother to newborns occurs without causing congenital forms of disease. [26]. There is no correlation between abs titres and the severity of the disease [27], although this fact is observed in other well established autoimmune diseases like myasthenia gravis. Up to now, no animal models of disease are available. [28] Moreover GAD abs neurological syndrome responds to immunotherapy less than diseases recently described in association with Neuronal Surface antibodies (NSabs), like anti-NMDAR or anti-LGI1[23].

Several studies in vitro and in vivo, have addressed the issue of the pathogenic role of GAD abs (Table 1).

<table>
<thead>
<tr>
<th>Study</th>
<th>Main findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinkel, 1998 [17]</td>
<td>Inhibition of C-terminal segment of the GAD by a non-competitive mechanism in SPS patients (rat cerebellar extracts) and not in T1DM</td>
</tr>
<tr>
<td>Ishida, 1999 [29]</td>
<td>Ig from serum and CSF from a CA patient suppresses GABAergic suppression in rat cerebellar slices</td>
</tr>
<tr>
<td>Mitoma, 2000 [30]</td>
<td>CSF Ig from an ataxic patient decreased GABA release onto Purkinje cells</td>
</tr>
<tr>
<td>Takenoshita, 2001 [31]</td>
<td>In rat cerebellar slices exposition to serum or CSF from patients with CA produced a gradual decreased of the inhibitory postsynaptic currents in Purkinje cells</td>
</tr>
<tr>
<td>Vianello, 2008 [32]</td>
<td>Increase in postsynaptic inhibitory potentials in hippocampal cultured neurons exposed to GAD abs</td>
</tr>
<tr>
<td>Manto, 2007 [28]</td>
<td>Intracerebellar and paraspinal injection of IgG from SPS patients induced continuous motor activity in rats</td>
</tr>
<tr>
<td>Manto, 2011 [33]</td>
<td>Abs from SPS patients increased glutamate levels and inhibit GAD activity in rats; Abs from patients with cerebellar ataxia had no effect on glutamate levels</td>
</tr>
<tr>
<td>Hampe, 2013 [34]</td>
<td>Intracerebellar injection of GAD abs against some specific epitopes determined impaired procedural spatial functions in rats</td>
</tr>
<tr>
<td>Hansen, 2013 [35]</td>
<td>Intraventricular injections of purified IgG GAD abs from a SPS induced stiffness-like behavior</td>
</tr>
</tbody>
</table>

Table 1. Studies exploring pathogenicity of GAD abs

The open issue of the wide clinical spectrum (but a single antibody positivity) has been addressed in some recent studies. [36] In 2015, some authors explored the hypothesis that distinct GAD antibodies could elicit specific phenotypes, finding that antibodies present in cerebellar ataxia and SPS recognized an epitope distinct from T1DM and limbic encephalitis. In the same year Gresa-Arribas et al [16] did not identify differences in the GAD epitope repertoire among the main examined neurological syndromes but found higher titres of GAD65
abs in the CSF of patients affected by limbic encephalitis or cerebellar ataxia compared to those with SPS. This latter work did not find further surface antibodies like anti-gephyrin and GABARAP, which had previously been suggested as possibly having co-reactivity with more pathogenetic value [19]; moreover, only 11% of cases harboured other NsABs (GABAaR and GlyR) but no SPS patient had coexisting GlyR, as found elsewhere in 15% of patients. [37]

The pathogenetic role of GAD abs is supported also by the demonstration that SPS patients have intrathecal synthesis of GAD abs (more in cerebellar ataxia than in SPS [11] [38]) and GAD-specific oligoclonal bands in CSF [39], even if this notion has been criticized [40].

Pathophysiology of GAD abs related diseases
Experimental and clinical data strongly support the role of GABAergic dysfunction in neurological diseases associated with GAD abs. Improvement after administration of benzodiazepines or other GABA receptor agonists, like baclofen, is a feature of SPS patients that has been recognized since the earlier descriptions. Floeter et al [41] proved through the study of H-reflex that the clinical features of SPS were explained by the lack of spinal inhibitory GABAergic mechanisms.

In vitro studies, especially on rat cerebellar slices, have suggested that cerebellar ataxia could be mediated by selective impairment of presynaptic GABAergic transmission from cerebellar basket and stellate cells to Purkinje cells which project into the deep cerebellar and vestibular nuclei. Impaired GABAergic projection within the brainstem could explain the oculomotor dysfunction common in these subjects. [42]

Reduction of GABA levels were reported in GAD abs positive patients affected with temporal lobe epilepsy; [43] this could lead to impairment of GABAergic inhibitory tone determining epileptic manifestations. [44]

Pathology
Pathological studies in SPS show chromatolysis and vacualisation of the anterior horn cells and a loss of alpha-motor, gamma-motor neurons and spinal interneurons with gliosis. Perivascular lymphocytic cuffing in different CNS regions has been shown in PERM and SPS with brainstem signs. [12] In cerebellar ataxia, loss of Purkinje cells with preservation of basket cells in the molecular layer has been shown. Proliferation of the Bergmann glia has also been reported. [45] [46]. Loss of pyramidal cells and inflammatory infiltrates have been reported in patients affected by limbic encephalitis with high titres of GAD abs in serum. [47]

Laboratory detection of GAD abs
Several methods are routinely used in clinical practice to detect GAD abs: immunohistochemistry (IHC), radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). More rarely and for research purposes immunoprecipitation assay and radiobinding assay are also used. [48] Notably, to date there are no assays that unambiguously distinguish GAD abs specific for neurological diseases rather than diabetes. [11]
a) Immunohistochemistry
IHC is considered the most specific technique for detecting GAD abs in neurological patients. Reactivity should be confirmed by RIA or another equivalent technique, according to general recommendations for the detection of antibodies in neurological patients. [49] IHC is positive only in samples with high-titre GAD abs (> 2000 IU/ml according to Saiz 2008 [11].
Paraformaldehyde-fixed frozen sections of rat cerebellum are incubated with serial dilutions of sera and/or CSF. The sections are incubated with peroxidase- or fluorescein-conjugated rabbit anti-human immunoglobulin and the reaction is usually developed with diaminobenzidine tetrahydrochloride and hydrogen peroxidase. [50] Positive sera stain the axon hillocks of Purkinje cells and diffuse nerve terminals in the molecular and granular layers of the cerebellum.

b) Western Blot
Total homogenate of human cerebellum is separated by sodium dodecylsulphate gel electrophoresis and transferred to nitrocellulose membrane. Strips are incubated with patients’ sera and polyclonal GAD abs (positive control) and then exposed to peroxidase-conjugated rabbit anti-human immunoglobulin. Immunoblot can also be performed using recombinant human GAD65 transferred to nitrocellulose; [48] some commercial kits use this system (dot-blot).

c) Radioimmunoassay
Serum samples are incubated with 125I-labelled human rGAD-65 and protein A-Sepharose is added. After centrifugation the precipitates are counted for 125I with a gamma scintillation counter. The results are interpolated in the standard curve constructed using dilution of a positive control serum. [51]

d) Enzyme-linked immunosorbent assay
In recent years ELISA has been replacing RIA for its practical advantages. These assays are performed using recombinant human GAD65 reacting with serum developed with a colorimetric method and using a plate reader to calculate optical density. Most commercial ELISA used to diagnose GAD abs neurological diseases are validated only for diabetic patients and designed to detect antibodies at low titres; moreover they sometimes do not use standardized units. Considering their important role in the clinical classification of diabetes, the Immunology of Diabetes Society (IDS) and the US Centers for Disease Control and Prevention, established a multicentre collaboration to improve and standardise the measurement of the autoantibodies predictive of type 1 diabetes, included GAD abs. This was achieved by determining a standard unit of measure (International Units) and by periodic standardisation workshops. [52]
Clinical spectrum of GAD abs related neurological diseases: stiff person syndrome

SPS is a rare disease (prevalence has been estimated at 1 in 1,250,000) initially described in men, but is more frequent in women (70%) [53]. Usually the disease starts in the mid-to-late 30s. [54] The first cases of “stiff man syndrome” were described in 1956, but the term was then replaced in 1991 by “stiff person syndrome” on the basis of the higher incidence in women. [55]

The disease tends to have an insidious onset (but in some series, acute and subacute have been frequently reported [53]) with symmetric rigidity or hypertonia due to co-contraction of antagonist muscles causing slow motion (“robotic gait”) resembling parkinsonism. Muscle hypertrophy and abnormal postures are frequent, leading to lumbar hyperlordosis. Stiffness extends from the trunk to the proximal and then distal limbs, determining gait disturbances with falls. Painful muscular spasms and startle response are other frequent findings; these phenomena are fluctuating and triggered by emotional upset and by tactile, auditory and visual stimuli. Moreover, exteroceptive or cutaneousmuscular reflexes are enhanced and spread into muscles normally not involved in the reflex. [56] Chest spasms can determine respiratory disturbances in some cases. Rigidity improves during the sleep. [27] Brainstem manifestations include oculomotor disturbances like dysconjugate gaze, horizontal and vertical supranuclear gaze palsy, hypometric and slow saccades, impaired smooth pursuit, nystagmus and abduction deficits. Brisk reflexes are a frequent sign. Dysautonomic crisis with tachycardia, blood pressure instability and hyperthermia are reported. [57]. Psychiatric comorbidity is frequent: depression, anxiety, alcohol abuse and specific phobias are reported. Weakness and sensory disturbances are not usually part of the classical spectrum of SPS but other neurological signs can be present. [12] The disease usually has a chronic course and after a slow progression the condition stabilizes for years or decades. [56]

Clinical variants of SPS are also described, in particular focal SPS, jerky SPS, progressive encephalopathy with rigidity and myoclonus (PERM) and paraneoplastic SPS (see below).

Focal SPS often localizes to one of the lower limbs (stiff limb syndrome). The disease can be limited to that segment for many years or can spread to other districts, resembling the classical form of SPS. [58] When the upper limbs are involved paraneoplastic SPS should be suspected.

When SPS presents with prominent myoclonus and jerking movements, it is termed “jerking SPS”. Myoclonus is also one of the prominent features of PERM that manifests in patients older than classical SPS patients (50-60 years). This form of disease has a slow onset (but subacute forms are increasingly described) of proximal and distal rigidity, oculomotor dysfunction, dysphagia and ataxia due to brainstem involvement, dysautonomia, axial and limb rigidity with profuse sweating. Cranial nerve, brainstem and long tract signs involvement differentiates PERM from SPS. [56] Recently detection of Glycine receptor antibodies [59] in these patients, with or without the co-presence of GAD antibodies, suggests that PERM could be a distinct disease.
Diagnosis of SPS is based on clinical and electrophysiological data. Electromyography shows continuous motor-unit activity with normal morphology including at rest and during contraction of antagonist muscles, which normally causes relaxation of the segment. [54] This activity is better found in axial muscles (thoracolumbar paraspinal and rectus abdominis muscle). No sign of denervation is usually found and nerve conduction velocity is normal [56].

Brain MRI is usually normal and can be helpful in differential diagnosis. CSF often shows signs of inflammation; oligoclonal immunoglobulin bands (IgG) are found in about 70% of cases; [60] less frequently an increase of cell and protein content is also encountered. High-titre serum GAD abs are considered supportive features, and their positivity is classically reported in 60% cases. This incidence varies according to clinical inclusion criteria and methods of detection. Their presence can be also found in CSF with intrathecal synthesis documented in the majority of patients when appropriately searched for. [39]

The therapeutical approach is based on drugs enhancing GABAergic transmission, like diazepam (Type-A GABA receptor agonist) or baclofen (Type-B), and immunotherapy. Benzodiazepines alleviate stiffness and painful spasms; this fact can be considered as confirmation of diagnosis. Higher doses of benzodiazepines can be required during the course of the disease causing drowsiness which limits their use. Botulinum toxin and intrathecal baclofen have been used with frequent adverse reactions but without long-term benefit. [61]

The benefit from immunotherapy has been demonstrated. Intravenous immunoglobulin (IVIG) showed efficacy in a randomized trial [62]. Plasma-exchange has efficacy, although results vary [63] [64] [65], and it can also be used as maintenance therapy [66]. More recently, anti-B cell therapies with rituximab have been proposed but a recent double-blind placebo-controlled trial failed to show differences versus placebo in stiffness score at 6 months. [67]

Cerebellar ataxia

Cerebellar ataxia (CA) associated with GAD abs is a rare disease accounting for about 2% of patients affected by sporadic ataxia. [68] It is probably the second most frequent disease associated with GAD abs. [69] [23] It most frequently affects women from the fifth or sixth decades of life. Rare familial cases have been reported in association with other autoimmune conditions. [70] Autoimmune comorbidity is very frequently reported in GAD abs CA, patients being affected with T1DM, thyroiditis, pernicious anaemia, polyglandular autoimmune syndrome, myasthenia and vitiligo. [71] CA can be paraneoplastic in about 10% of patients, with small cell lung carcinoma, thymoma and breast cancer being more frequently described. [72]

CA tends to have a slow or insidious onset, but a subacute onset is also frequently reported. [42] In about 25% of patients transient vertigo or other neurological dysfunctions affecting the brainstem, like diplopia or dysarthria can occur in a prodromic phase. [23] Clinical manifestations consist chiefly of moderate/severe gait ataxia, limb ataxia (usually asymmetric), dysarthria, kinetic tremour and oculomotor deficits, namely central nystagmus.
Overlapping signs SPS-like have been reported, manifesting as muscle rigidity and spasms affecting especially the lower limbs. Moreover, also epilepsy can be associated in 13% of patients. [16] Usually the disease has a progressive course and can be disabling. Magnetic Resonance Imaging (MRI) of the brain can show mild atrophy especially a few years after the beginning of the illness. Diagnosis is made after careful exclusion of other causes, particularly genetic causes, and on the basis of GAD abs of high titre in the serum and/or CSF.

Immune therapy generally consists of steroids and immunoglobulin; plasma-exchange, Rituximab and Mycophenolate have also been reported. Therapy has efficacy in about 50% of patients; improvement after immunotherapy is more common in patients with subacute onset. [23]

**Epilepsy**

Epilepsy was reported in association to SPS in the earlier descriptions; the first patient harbouring GAD abs described by Solimena had coexistent epilepsy. [8] Temporal lobe epilepsy was then established in association with GAD abs. [73] Globally, the frequency of GAD abs in epileptic patients is about 1-5%, [74] [75] although methods of detection and inclusion criteria are variable. Dubey in a consecutive recent series of patients with epilepsy of unknown etiology found 8% of patients harbouring high titer GAD abs (> 20 nmol/L) [76]

Adult onset of temporal lobe epilepsy seems the phenotype that is associated with the finding of high titers GAD abs. [74] Some studies have focused on the resistance to anti-epileptic drugs and long duration [75] [77]

These patients are more frequently women with autoimmune comorbidity. They have no clinical and radiological features of limbic encephalitis, in particular they may have a chronic onset and usually a negative brain MRI. Epilepsy is also found as a feature of other GAD-abs-associated diseases, like stiff person syndrome or cerebellar ataxia.

There are not sufficient data to assess the benefit of immunotherapy in GAD abs epilepsy; in the series by Quek et al 2012, [78] 3 out of 5 GAD65-seropositive patients (60%) reported benefit from immunotherapy with variable regimens. In the study by Toledano et al. 2014 there was a high representation of GAD positive patients in a non responder group to immunotherapy for patients with presumed autoimmune epilepsy. [79] Some authors suggest the benefit from chronic immune-suppression rather than “first line” therapy. [74]

**Limbic encephalitis**

GAD abs have recently been described in encephalitis with involvement of limbic areas. [47] Non-infectious limbic encephalitis (LE) is an inflammatory clinico-pathological entity that can be paraneoplastic or non paraneoplastic. [80] In the last ten years several neural antibody markers have been described; they are classified on the basis of their antigenic target in intracellular and membrane antibodies. [81]

In recent years growing evidence has been accumulated that GAD abs can also associated with LE. Malter in 2010 found GAD abs in 17% of a cohort of patients collected prospectively. Patients developed a chronic form
of inflammation of the temporal lobes with prominent epileptic features; they were younger and more frequently women as compared to patients with Voltage-Gated Potassium Channel-abs (VGKC) LE. They were resistant to immunotherapy with poor memory outcome. [47] In the series of GAD abs neurological patients reported by Saiz, LE were described in two cases (females aged 47 and 49) and in one case of paraneoplastic origin in a 70-year-old male patient. [11] Five other patients affected by paraneoplastic LE were described by Arino (see below). [82]

From a clinical point of view, LE associated with GAD abs present with the classic triad of memory deficit, psychiatric symptoms and epileptic seizures, which have recently also been listed in the clinical criteria. [83] No specific clinical findings have been found in GAD-abs LE. Similarly to the other diseases, co-existence with other autoimmune diseases like T1DM and thyroiditis has been frequently reported.

Brain imaging by MRI shows the typical hyperintensity of mesio-temporal lobes, without contrast enhancement. Compared with LE associated with neuronal surface antibodies, response to therapy is poorer; in a recent review of 58 published cases, only 4 were noted as fully responding to treatment.

**Paraneoplastic diseases**

SPS can be diagnosed in association with systemic neoplasms in less than 10% of cases. In these cases amphyphisin antibodies can be found, although in a minority of patients. Typically they are women affected by breast cancer, in whom low titres of GAD abs can coexist [84]. Differently from classic SPS, the neck and upper limbs are more frequently involved. Paraneoplastic SPS have also been described in association with isolated GAD abs and anti-Ri abs. [85] The associated tumours most frequently reported have been thymomas and breast cancer. Notably, PERM can also be paraneoplastic, e.g. it can be found in association with small cell lung cancer (Kyskan et al., 2013) and breast cancer [82].

In the retrospective series reported by Arino in 2016, [82] when GAD abs occurred in association with limbic encephalitis or other classical paraneoplastic syndromes (cerebellar degeneration, opsoclonus-myoclonus syndrome or paraneoplastic encephalomyelitis), with or without the co-existence of further antineuronal antibodies, the risk for cancer is higher than in patients with SPS or cerebellar ataxia (in particular lung cancer). Generally, paraneoplastic GAD autoimmunity is infrequent but in particular cases, oncological screening should be performed, especially in the presence of a classical paraneoplastic neurological syndrome. [82]

**Association with autoimmune diseases**

As different from autoimmune neurological diseases associated with other neural antibodies, GAD-abs-related diseases frequently coexist with other autoimmune diseases and this association was underlined in earlier reports [56]. T1MD, thyroiditis, pernicious anaemia, vitiligo are frequently associated, especially with SPS and cerebellar ataxia. Organ-specific antibodies are also a common finding, with or without the clinical manifestation of the disease.
3. AIM OF THE STUDY

This project consists of a retrospective observational study describing a cohort of GAD abs seropositive patients affected with neurological diseases, whose samples were tested by an optimized commercial assay. The study aims to:
- explore the clinical spectrum of GAD abs-related diseases, comparing data with literature;
- to perform a clinical validation of a modified commercial ELISA kit to detect GAD abs, also at high levels, in serum and CSF of patients with suspected neurological autoimmune diseases;
- to establish a threshold of clinical relevance for serum GAD abs determined by ELISA;
- to clarify the meaning of serum GAD abs at low titers in neurological patients;
- to assess if GAD abs coexist with other known antibodies.
METHODS

Subjects
We tested 330 patients with suspected autoimmune neurological disease, whose samples (serum and CSF) had been sent to our Laboratory between 2009 and 2015 for the detection of GAD abs. Patients with positive serum, available clinical information and at least 3 months follow-up were included in the study.
Clinical information on positive patients was obtained retrospectively as part of clinical routine, from the referring physician through a structured questionnaire sent by e-mail.
Analysis also included 215 consecutive samples from patients with suspected T1DM or polyglandular autoimmunity (whose neurological status was unknown), tested between September 2016 and September 2017. Moreover, 45 control sera from people affected with various neurological diseases and 8 from healthy people were included in the study.

Review of clinical data
One external physician, blind to GAD abs titration and to other antibody results, analyzed the collected clinical data. Firstly he reviewed the final diagnosis of the case and established whether it fitted with one of the four more frequently described clinical phenotypes of GAD abs, after careful exclusion of alternative causes: SPS (and variants), cerebellar ataxia, encephalitis and epilepsy. [11] He then had to judge whether the features of the case resembled typical features of GAD abs from the commonly recognized clinical spectrum, labeling the case as “typical” (Figure 2).

![Figure 2. Clinical revision process to assess typical patients](image)

Analysis of the external observer was arbitrarily based on the following core features, according to published data:
a) stiff person syndrome [86]:
- insidious onset of muscular rigidity of the limbs and axial (trunk) muscles with superimposed spasms
- continuous co-contraction of agonist and antagonist muscles with inability to relax
b) cerebellar ataxia [42]
- subacute or slow onset of gait ataxia and oculomotor abnormalities (nystagmus)
- normal or cerebellar atrophy without brainstem atrophy
- autoimmune comorbidity (see below)
c) encephalitis [83]
- subacute onset of working memory deficits, seizures, or psychiatric symptoms suggesting involvement of the limbic system
- bilateral brain abnormalities on T2-weighted fluid-attenuated inversion recovery MRI, highly restricted to the medial temporal lobes
- EEG with epileptiform discharges or slow wave-activity involving the temporal lobes (ictal or interictal)
d) epilepsy
- slow or acute onset of focal temporal epilepsy
- drug resistant epilepsy
- negative brain MRI
- EEG with epileptiform discharges or slow-wave activity involving the temporal lobes (ictal or interictal)
The following were supportive features used to consider the case as typical:
- signs of inflammation in the CSF (pleocytosis, increased protein content or presence of oligoclonal bands)
- presence of other autoimmune comorbidities (especially for cerebellar ataxia)
- response to immunotherapy

ELISA

Commercial ELISA kits (RSR Limited) with an automated system (DSX, Technogenetics) were used. Reagents were allowed to stand at room temperature for at least 30 minutes before use. In each well 25 µl patient serum (or an equal amount of CSF), calibrators and controls were deposited. After covering the frame, the wells were shaken for 1 hour at room temperature. The samples were then aspirated and the wells washed three times with the diluted wash solution. In each well 100 µl of a solution of biotin conjugated with GAD65 were pipetted and incubated for one hour at room temperature. The wells were washed three times with the washing solution. Afterwards, 100 µl of streptavidin peroxidase solution were pipetted into each well and incubated for 20 minutes at room temperature. The wells were washed three times with the washing solution. Then, 100 µl of TMB (peroxidase substrate) solution were pipetted into each well and incubated for 20 minutes at room temperature without agitation. 100 µl of blocking solution were added and incubated for 5 seconds under agitation. Within 10 minutes, the absorbance reading was performed in each well at 405 nm. The results of calibrators were plotted in a graph representing the logarithmic scale of the concentration versus the absorbance value. Based on this graph it was possible to obtain the calibration curve and the samples concentration according to the range of the kit (5 – 2000 IU/ml).
**Estimation of titres (modified ELISA)**

For concentration values above 2000 UI/mL an algorithm was applied through serial dilutions at 1:10, 1:320, 1:640 and 1:1280 for serum; 1:10, 1:80 and 1:160 for CSF. These dilutions were established arbitrarily according to preliminary experiments.

Applying a recently proposed model [87] it was possible to calculate the estimation of the sample concentration on the basis of the single results obtained from the serial dilutions. We established that at least one of the results was included in the semilinear portion of the sigmoid calibration curve (see below), arbitrarily established in a range of ± 0.5 OD compared to the sigmoid flex point; when the point was not inside this range, an intermediate or higher dilution was performed.

The ELISA kit gives the concentration value (range 5 – 2000) starting from an absorbance value, based on the relation between concentration and absorbance expressed in the following formula (graphically expressed as a sigmoid curve).

\[
E(Y) = b_2 + \frac{b_1 - b_2}{1 + \left(\frac{x}{b_3}\right)^{b_4}}
\]

where \(E(Y)\) is the absorbance revealed by the machine at the concentration of antibody \(x\). The parameters \(b_1, b_2, b_3, \) and \(b_4\) are fixed parameters varying in each experiment; they represent, respectively, the inferior and superior asymptotes of the curve, the antibody concentration at the level of absorbance (average compared to the two asymptotes) and the curve grade (Figure 3). \(b_1, b_2, b_3, \) and \(b_4\) are calculated by the machine at the beginning of each experiment, through a calibration curve starting from samples with known concentration provided by the manufacturer. Considering the geometry of the sigmoidal curve, it is possible to observe that close to the asymptote, small absorbance variations correspond to very different concentration values. For this reason, the precision of the instrument is not constant in determining the antibody concentration, but it is higher at middle absorbance levels and lower when the absorbance is near the asymptote levels. Therefore, the arithmetic average of multiple dilutions is not precise, rather it is preferable to calculate a ponderate average, based on the position of the result in the sigmoidal curve.
Figure 3. Sigmoidal curve with parameters b1, b2, b3 and b4

The ponderate average was calculated according to Cheung et al [87] as follows. For sample $i$, $x_i$ is defined as the antibody concentration of the sample, $x_{ik}$ is the concentration of the sample diluted $K$ times, $dil_k$ is the dilution factor, and $y_{ik}$ is the absorbance revealed by the machine for the sample diluted $K$ times.

The natural logarithm of the $x_{ik}$ concentration for each $k$ is calculated as follows:

$$\ln( x_{ik} ) = \ln( b_1 ) + \ln( dil_k ) + \frac{1}{b_4} \times \ln \left( \frac{b_1 - y_{ik}}{y_{ik} - b_2} \right)$$

Then the weighted factor $w_{ik}$ is calculated for each $k$:

$$w_{ik} = \frac{1}{\left( \frac{1}{b_1 - y_{ik}} + \frac{1}{y_{ik} - b_2} \right)^2}$$

Lastly, the natural logarithm of the concentration of undiluted sample is calculated:

$$\ln( x_i ) = \frac{1}{\sum_{k=1}^{K} w_{ik}} \times \sum_{k=1}^{K} w_{ik} \times \ln( x_{ik} )$$
Therefore the antibody concentration of the sample not diluted can be obtained by the following formula:

\[ X_i = e^{\ln(y_i)} \]

To speed up the mathematical calculation, an algorithm was created and developed in a computer programme (Dr. A. Brieda, dr. S. Bellio).

**Intrathecal synthesis calculation**

When paired serum and CSF samples were available, the Link index (IgG index) and antibody index of GADA (AI\_GAD) were calculated, as indicators of IgG intrathecal synthesis and GAD abs intrathecal synthesis, respectively. For this purpose, the nephelometric dosage (Siemens) of albumin and IgG were performed on both serum and cerebrospinal fluid.

The Link index was calculated through the following formula:

\[ \text{IgG INDEX} = Q_{\text{IgG}} + Q_{\text{Alb}} \]

that is

\[ \text{IgG INDEX} = \frac{Q_{\text{CSF}}}{Q_{\text{Serum}}} \cdot \frac{Q_{\text{Alb}}}{Q_{\text{CSF}}} \]

A value above 0.7 is suggestive of IgG intrathecal synthesis.

The AI\_GAD was calculated by the following formula:

\[ \text{AIGAD} = Q_{\text{GAD}} + Q_{\text{IgG}} \]

\[ \text{AIGAD} = \frac{Q_{\text{CSF}}}{Q_{\text{Serum}}} \cdot \frac{Q_{\text{Alb}}}{Q_{\text{CSF}}} \]

A value of AI-GAD higher than 1.5 is suggestive of intrathecal synthesis of GADA according to the normal range for AI. [88] When IgG are predominantly synthesized intrathecally, that is when Q\_IgG \( >\) Qlim, the following expression is used to better estimate AI

\[ \text{AIGAD} = Q_{\text{GAD}}/Q_{\lim} \]

where the limit ratio function (Qlim) is considered:
Inter-assay and intra-assay precision

To evaluate the intra-assay repeatability of ELISA, three dilutions of the same sample were tested four times in the same experiment; the dilutions (1:320, 1:640 e 1:1280) were performed by four different operators, using the same instruments. The variation coefficients (CV) were evaluated using the following formula:

\[ CV = \frac{\text{standard deviation of the 4 titration s}}{\text{average of the 4 titrations}} \times 100 \]

To evaluate the inter-assay reproducibility of ELISA, the same serum samples were tested in four different experiments performed on different days. One serum sample (high GAD abs titre) was diluted at 1:80, 1:320, 1:640 and 1:1280. The other samples (patients cohort with lower titre) were tested 1:1. The variation coefficient (CV) was calculated using the same expression.

Indirect immunohistochemistry

Serum and CSF were screened by IHC performed on rat cerebellum frozen in 7 µm-thick sections. Rat cerebellum was fixed in paraformaldehyde (PFA 2%) for 6 hours. The sections were incubated using patients' CSF (1:2) or serum (1:500) for 3 hours at 37°C. After two consecutive PBS washes, the sections were incubated with IgG-biotinylated for 30 minutes at room temperature. Reaction was obtained using the avidin-peroxidase reagents (ABC Vector reagent) and then developed by DAB staining (Dako). The slides were dehydrated using alcohol gradient (70%-96%-96%-100%-100% ethanol and twice washed in xylol). The slides were mounted with hydrophobic mount medium (Eukitt). The slides were observed at the optical microscope and the results were independently evaluated by two different operators.

Immunoblot

A commercial kit (Euroimmun) was used to test the serum samples for GAD and onconeural abs. All the reagents were kept at room temperature (RT) before using. The washing buffer (10X concentrated) and enzymatic conjugate (10x) were correctly diluted. Each strip used for the reaction was located in the respective channel and with the number clearly visible. Each channel was filled with 1.5ml of dilution buffer, incubated 5 minutes in agitation, after which the liquid was discarded. Each channel was then filled with 1.5ml of diluted sample (serum 1:100, CSF 1:5) and incubated at RT in agitation. The liquid was discarded and each strip was washed 3 times for 5 minutes each with 1.5ml of washing buffer in agitation. 1.5ml of diluted conjugate were added in each channel (human IgG conjugated with alkaline phosphatase) and incubated 30 minutes at RT in agitation. The strips were then washed as mentioned before. 1.5ml of substrate solution were added and
incubated for 10 minutes at RT in agitation. The substrate was then discarded and each strip was washed 3 times for one minute with distilled water. The strips were then located in a reference scheme, left to dry at room temperature and the results were read by an expert operator.

**Cell based assay**

The neuronal surface antibodies were screened using indirect immunofluorescence on commercial (Euroimmun) fixed cell lines transfected with vectors expressing the interested antigens (cell-based assay). Briefly, the slides contained biochips transfected with cDNA codifying the NMDAR, AMPAR, CASPR1, LGI1 or GABAbR antigens. The diluted serum (1:10 with Tween-PBS) and/or non-diluted CSF were incubated for 30 minutes at room temperature. The slides were washed for 5 minutes at room temperature in an agitator. The slides were then incubated with the secondary antibody (human IgG conjugated with FITC). After a second wash, the slides were mounted with glycerol and observed under the fluorescence microscope (Zeiss Aziopohlt) at 20X e 40X. The results were independently evaluated by two different operators and defined as “positive” or “negative” when the results were in accordance, otherwise the test was repeated.

**Statistical analysis**

To evaluate the accuracy of the ELISA test and establish the best cut off, a receiver-operator characteristic (ROC) analysis was performed, after determining the true positive cases on the basis of independent clinical analysis (typical GAD abs clinical spectrum). The Youden index was applied to find the threshold for higher accuracy (confidence intervals 95%).

Statistical analysis was then applied to the differences between groups using Fisher’s exact test or the Chi-square test for categorical variables, Student’s T test and the Mann-Whitney U for quantitative variables, where appropriate. Analysis was performed using R statistical software. P-values < 0.05 were considered significant.
RESULTS

Subjects

Forty-three patients tested positive for serum GAD abs by optimized ELISA (range 6–2019681 UI/ml), 35 with available clinical information were included in the study. They were 14 males and 21 females, age range 15-82 years old with the following final reviewed diagnosis: encephalitis (10), SPS (9), ataxia (5), epilepsy (2) and other diseases (9). The cases were all reviewed by an independent observer in order to assess the presence of typical features resembling GAD autoimmunity as described above. All patients with a diagnosis other than SPS, ataxia, epilepsy or epilepsy were excluded to this further clinical analysis and labeled immediately as “Atypical”. In 26/35 patients (74%) the final diagnosis belonged to the already reported spectrum of GAD-abs related diseases, specifically 10 patients diagnosed with encephalitis, 9 with SPS, 5 with ataxia and 2 with epilepsy. Among these, 12/26 failed to fit the criteria described in the methods and were noted as “Atypical”. 14 patients showed typical features; they were 9 cases with a final diagnosis of SPS, 3 cases affected by encephalitis and 2 patients affected by ataxia. No patient diagnosed with epilepsy was noted as “Typical”. Table 2 reports the results of this two-step analysis; patients are listed by GAD-abs titre in descending order.

<table>
<thead>
<tr>
<th>Patient</th>
<th>GAD-abs titre</th>
<th>Final diagnosis</th>
<th>GAD-abs related syndrome?</th>
<th>Typical features?</th>
</tr>
</thead>
<tbody>
<tr>
<td>6#</td>
<td>2019681</td>
<td>SPS</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>8#</td>
<td>1373559</td>
<td>Encephalitis</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>9#</td>
<td>1228265</td>
<td>SPS</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>10#</td>
<td>1165654</td>
<td>Epilepsy</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>14#</td>
<td>643289</td>
<td>Ataxia</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>13#</td>
<td>514435</td>
<td>SPS</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>5#</td>
<td>229049</td>
<td>Ataxia</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>7#</td>
<td>226862</td>
<td>SPS</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>4#</td>
<td>187202</td>
<td>Dysartria</td>
<td>NO</td>
<td>/</td>
</tr>
<tr>
<td>12#</td>
<td>98841</td>
<td>Encephalitis</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>11#</td>
<td>92028</td>
<td>SPS</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>1#</td>
<td>75738</td>
<td>Ataxia</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>3#</td>
<td>53993</td>
<td>SPS</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>15#</td>
<td>51475</td>
<td>Ataxia</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>2#</td>
<td>19440</td>
<td>Encephalitis</td>
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<td>YES</td>
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<tr>
<td>35#</td>
<td>6547</td>
<td>Corticobasal degeneration</td>
<td>NO</td>
<td>/</td>
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<tr>
<td>16#</td>
<td>1182</td>
<td>Encephalitis</td>
<td>YES</td>
<td>NO</td>
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<tr>
<td>34#</td>
<td>258</td>
<td>Chronic pain</td>
<td>NO</td>
<td>/</td>
</tr>
<tr>
<td>21#</td>
<td>224</td>
<td>Morvan syndrome</td>
<td>NO</td>
<td>/</td>
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<tr>
<td>19#</td>
<td>219</td>
<td>SPS (PERM)</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>29#</td>
<td>169</td>
<td>SPS</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>27#</td>
<td>49</td>
<td>Encephalitis</td>
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<td>32#</td>
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<tr>
<td>20#</td>
<td>39</td>
<td>SPS (PERM)</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>25#</td>
<td>39</td>
<td>Ataxia</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>17#</td>
<td>29</td>
<td>Encephalitis</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>28#</td>
<td>27</td>
<td>Encephalitis</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>
Typical patients were in the great majority women (78%) with a mean age of 50.9 years old (Table 3). Oligoclonal bands were one of the supportive data to note the patients as “typical” and were present in 64% of typical patients but also in 14% of atypical patients. Patients were treated with immunotherapy in a high proportion of cases (27/35); but typical patients more frequently underwent immune-suppression (9/14 versus 2/21; p < 0.001).

Assessment of the clinically relevant cut-off

On the basis of the clinical assessment performed as showed above (typical versus atypical cases), a ROC curve was built in order to assess the cut-off for the clinical relevance of serum GAD abs detected by our assay (Figure 4).

<p>| | | | | |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>30#</td>
<td>26</td>
<td>Parkinsonism</td>
<td>NO</td>
<td>/</td>
</tr>
<tr>
<td>26#</td>
<td>24</td>
<td>Chronic pain</td>
<td>NO</td>
<td>/</td>
</tr>
<tr>
<td>18#</td>
<td>21</td>
<td>Wernicke</td>
<td>NO</td>
<td>/</td>
</tr>
<tr>
<td>23#</td>
<td>18</td>
<td>Motor neuropathy</td>
<td>NO</td>
<td>/</td>
</tr>
<tr>
<td>31#</td>
<td>14</td>
<td>Chronic pain</td>
<td>NO</td>
<td>/</td>
</tr>
<tr>
<td>22#</td>
<td>11</td>
<td>Encephalitis</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>33#</td>
<td>11</td>
<td>Epilepsy</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>24#</td>
<td>6</td>
<td>Encephalitis</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>

Table 2. Final diagnosis and assessment of typical features by the external observer
The area under the curve (AUC) was 0.842 (standard error 0.66; 95 confidence interval 0.713 – 0.971). Calculation of the best cut off according to the Youden method [89] was 12993 UI/ml. This cut off identifies two groups of patients with either high-titre (> 12993 IU/ml) or low-titre (< 12993 IU/ml) antibodies.

According to this cut off, the test had a sensitivity = 78.57% (CI 95% 57.08% – 100.07%), specificity = 80.95% (64.16% - 97.75%), positive predictive value = 73.33% (50.95% - 95.71%), negative predictive value = 85% (69.35% - 100.65), odds ratio = 15.583 (2.90 – 83.46).

**Control groups**

53 subjects were tested as controls: 45 were patients affected with neurodegenerative diseases, 14 demyelinating diseases and 6 neuropathy; 8 were healthy volunteers. All controls tested negative for GAD abs.

From October 2016 to October 2017, 215 samples sent for suspected endocrinological diseases (T1DM or polyglandular autoimmune syndromes) were tested by ELISA; a total of 43 samples tested positive; 6 (14,0%) had a titer between 2000 – 13000 IU/ml; in 2/43 cases (4,7%) GAD abs were higher than the aforementioned threshold (65134 and 405093 IU/ml). The neurological status of these subjects is unknown.
Serum GAD abs titer range in typical patients was between 39 – 2019681 IU/ml (mean = 453325) significantly higher than atypical patients (range 6 – 1165654, mean = 78186) (p < 0.001) and endocrinological group (range 6 – 405093, mean = 12069) (p < 0.0001); titers of atypical were not significantly different from endocrinological group (p = 0.33) (Figure 5).

**Stiff person syndrome**

Patients with a final diagnosis of SPS (Table 3) were 6 females and 3 males (age range 42 – 82, mean 59.3). All 6 females had serum GAD abs with high titres (53993 – 2019681 IU/ml). The 3 males had titres ranging from 39 to 219 IU/ml and two of them had a final diagnosis of PERM. These two patients were also tested for Glycine receptor (GlyR) antibodies and one of them (patient 19#) was highly positive in the serum (1:800) and CSF. He was a 70-year-old man who developed severe encephalopathy with fever, behavioural changes, ocular dysfunction, craniofacial spasms, marked myoclonus and startle; he responded brilliantly to rituximab (weekly 375 mg/m² for 1 month) after failing with intravenous immunoglobulin. After therapy, the GlyR abs were undetectable in the serum but persisted in the CSF; the GAD abs titre also decreased from 219 to 24 IU/ml.

Five out of 9 patients had comorbidity with other autoimmune diseases. Except in one case, which showed a slight protein content increase, standard examination of the CSF was negative; the oligoclonal band pattern was found by IEF in 3/6 patients, whereas one patient had a mirror pattern of identical oligoclonal bands in the serum and CSF. All patients except patient 19# showed a response to symptomatic therapy with benzodiazepines and at least a partial response to immune-treatment was reported in all patients, although only patient #29 had a modified rankin scale score of 0 at the last follow up. Notably 5/9 patients underwent immune-suppression with
Rituximab (3), Azathioprine (2) and Cyclophosphamide (1).

<table>
<thead>
<tr>
<th>N. Pt</th>
<th>Sex; age at onset</th>
<th>GAD serum titer</th>
<th>Clinical features</th>
<th>CSF (IEF)</th>
<th>EMG</th>
<th>Autoimmune comorbidity; tumor; other neural antibody</th>
<th>Immunotherapy (response?)</th>
<th>Last Ms (months follow up)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6# F 42</td>
<td>2019681</td>
<td>Subacute onset of left leg weakness and rigidity; axial and limb rigidity with spasms; phobias</td>
<td>Normal (OCB)</td>
<td>MUP firing triggered by cutaneous stimulation.</td>
<td>Pernicious anemia; autoimmune thyroiditis.</td>
<td>Steroids, IVIG, PEX, Rituximab (YES)</td>
<td>2 (18)</td>
<td></td>
</tr>
<tr>
<td>13# F 51</td>
<td>514435</td>
<td>Insidious onset of axial and limb stiffness with superimposed spasms; anxiety</td>
<td>NA</td>
<td>Continuous MUP firing.</td>
<td>Autoimmune thyroiditis</td>
<td>IVIG, PEX, Azatyoprine (YES)</td>
<td>1 (150)</td>
<td></td>
</tr>
<tr>
<td>7# F 57</td>
<td>226862</td>
<td>Subacute onset of leg rigidity; falls; depression and anxiety</td>
<td>NA</td>
<td>NA</td>
<td>Vitiligo</td>
<td>Steroids, IVIG, PEX, Azatyoprine, Rituximab (YES)</td>
<td>4 (27)</td>
<td></td>
</tr>
<tr>
<td>9# F 65</td>
<td>122865</td>
<td>Slow onset of leg rigidity, than also right leg and trunkal stiffness; spasms; anxiety and depression.</td>
<td>Normal (OCB)</td>
<td>Continuous MUP firing; co-contraction of antagonist muscles</td>
<td>Pernicious anemia; Graves disease;</td>
<td>Steroids, IVIG (YES)</td>
<td>2 (18)</td>
<td></td>
</tr>
<tr>
<td>11# F 52</td>
<td>92028</td>
<td>Axial and limb stiffness; spasms; depression and anxiety; spinal deformity.</td>
<td>NA</td>
<td>Firing at rest in the paraspinal and proximal leg muscles.</td>
<td>No. Anti-amphiphysin.</td>
<td>IVIG, PEX (YES)</td>
<td>1 (86)</td>
<td></td>
</tr>
<tr>
<td>3# F 71</td>
<td>33993</td>
<td>Insidious onset of proximal weakness; axial and limb rigidity with spasms; anxiety</td>
<td>Normal (Negative)</td>
<td>Continuous MUP firing</td>
<td>Autoimmune thyroiditis.</td>
<td>Steroids, IVIG (YES)</td>
<td>NA (66)</td>
<td></td>
</tr>
<tr>
<td>19# M 70</td>
<td>219</td>
<td>Subacute onset of fever, behavior changes, dysarthria, dysphagia, cranio-facial spasms, cranial nerve deficits; myoclonus; startles and seizures (PERM)</td>
<td>Normal (Negative)</td>
<td>Spontaneous and reflexed myoclonus.</td>
<td>No. Anti-Glycine Receptor</td>
<td>IVIG, Rituximab (YES)</td>
<td>NA (24)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Clinical data of patients affected by Stiff Person Syndrome (MUP = Motor unit potential, NA = not available information, OCB = Oligoclonal Bands; Normal CSF = proteins < 45 mg/dl, leukocites < 5 /mm3)

<p>| | | | | |</p>
<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>29#</td>
<td>M 82</td>
<td>169</td>
<td>Subacute onset of trunkal and left leg rigidity; spasms</td>
<td>Protein content increase. Mirror pattern.</td>
</tr>
<tr>
<td>20#</td>
<td>M 44</td>
<td>39</td>
<td>Subacute onset of axial and lower limb stiffness; spasms; gait disturbances; anxiety; depression. (PERM)</td>
<td>27 leukocites; OCB +</td>
</tr>
</tbody>
</table>

Encephalitis

Patients with typical GAD encephalitis (n. 3) (Table 4) were females (age range 27 – 51) all with HT GAD abs (19440 – 1373559 IU/ml). They were three females with clinical features of limbic encephalitis and bilateral mesio-temporal hyperintensity (Figure 6). Patients 2# was a 51 female that developed limbic encephalitis in the context of previously diagnosed stiff person syndrome. Two CSF samples were available, and both were positive for oligoclonal bands, whereas cell and protein content was normal. 2/3 had altered electroencephalography and 2/3 had autoimmune comorbidity. Notably patient 12# was treated with rituximab; outcome of this patient was dominated by severe memory deficits reported also in patient 8#.

7/10 patients with atypical features were 4 males and 3 females (age range 15 – 68) and showed GAD Abs ranging from 6 to 1182 IU/ml. Clinical findings were more aspecific in comparison of typical patients; one patient had prominent brainstem involvement (patient 16#). Patient 28# had a LGI1 abs positive encephalopathy; patient #32 was a typical LGI1 abs positive patient with memory deficits and facio-brachial dystonic seizures that had a brilliant response to immunotherapy.

CSF showed signs of inflammation in 4 up to 7 cases; 2/5 available IEF showed oligoclonal bands. Brain MRI was altered in 3/7 cases with inflammatory involvement of thalamus (3 cases), brainstem and cerebellum. Electroencephalography showed pathological findings in 6/7 patients, only in one case suggesting involvement of temporal lobes (pt. 28#). 5/7 cases underwent immunotherapy and at least partial response was always reported. Only in patient 24# an immune-suppression was started with mycophenolate; he was a 64 years old man affected by non-Hodgkin lymphoma; he developed...
an encephalopathy with signs of inflammation on CSF some months after stem cell transplant.

<table>
<thead>
<tr>
<th>N. Pt</th>
<th>Sex; age at onset</th>
<th>GAD serum titer</th>
<th>Clinical features</th>
<th>CSF</th>
<th>MRI</th>
<th>EEG</th>
<th>Autoimmune comorbidity; tumor; other neural antibody</th>
<th>Immunotherapy (response?)</th>
<th>Last MrS (months follow up)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8#</td>
<td>F 32</td>
<td>137359</td>
<td>Acute onset of memory deficits, confusion and anxiety; generalized and temporal seizures, dystonia; depression</td>
<td>OCB +</td>
<td>Bilateral mesio-temporal hyperintensity</td>
<td>Normal (inter-ictal EEG)</td>
<td>Autoimmune thyroiditis, vitiligo, psoriasis</td>
<td>Steroids, IVIG (YES)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>12#</td>
<td>F 27</td>
<td>98841</td>
<td>After flu-like prodrome, acute onset of temporal seizures; memory deficit, psychosis with delusions and hallucinations; obsessive-compulsive behaviour.</td>
<td>OCB +</td>
<td>Bilateral mesio-temporal hyperintensity</td>
<td>Right temporal spike waves</td>
<td>Thyroiditis</td>
<td>Steroids, IVIG, Rituximab (YES)</td>
<td>Severe cognitive deficit (amnesia) (76)</td>
</tr>
<tr>
<td>2#</td>
<td>F 51</td>
<td>19440</td>
<td>Acute onset of temporal seizures; confusion and hallucinations; pre-existing limb stiffness with superimposed spasms</td>
<td>N.A.</td>
<td>Bilateral mesio-temporal hyperintensity</td>
<td>Diffuse spike-and-wave complexes</td>
<td>No</td>
<td>Steroids, IVIG, PEX (YES)</td>
<td>NA (28)</td>
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<tr>
<td>16#</td>
<td>M 35</td>
<td>1182</td>
<td>Subacute diplopia and disequilibrium</td>
<td>OCB +</td>
<td>Pontomesencephalic and bilateral thalamic hyperintensity</td>
<td>Normal</td>
<td>No</td>
<td>Steroids, IVIG (YES)</td>
<td>0 (24)</td>
</tr>
<tr>
<td>32#</td>
<td>F 68</td>
<td>68</td>
<td>Acute onset of facio-brachial dystonic seizures; cognitive impairment.</td>
<td>Normal (OCB -)</td>
<td>Normal</td>
<td>Left frontal slow waves</td>
<td>No. Anti-LGI1</td>
<td>Steroids, IVIG, PEX (YES)</td>
<td>0 (24)</td>
</tr>
<tr>
<td>27#</td>
<td>F 36</td>
<td>49</td>
<td>Insidious onset of memory deficit, apathy, vomiting headache; generalized seizures.</td>
<td>Normal, OCB</td>
<td>Normal</td>
<td>Slow frontal waves</td>
<td>No</td>
<td>Steroids, IVIG (YES)</td>
<td>0 (3)</td>
</tr>
<tr>
<td>17#</td>
<td>M 46</td>
<td>29</td>
<td>Acute onset of generalized seizures; tetraparesis.</td>
<td>Increased cells count; OCB NA</td>
<td>Right thalamus hyperintensity and corpus callosum</td>
<td>Anterior spike waves</td>
<td>No</td>
<td>NO</td>
<td>5 (3)</td>
</tr>
<tr>
<td>28#</td>
<td>F 67</td>
<td>27</td>
<td>Slow onset of complex temporal seizures, memory loss, depression</td>
<td>Normal (OCB NA)</td>
<td>Normal</td>
<td>Bitemporal spikes</td>
<td>Hashimoto thyroiditis, Anti-LGI1</td>
<td>Steroids, IVIG, PEX (YES)</td>
<td>NA (13)</td>
</tr>
<tr>
<td>22#</td>
<td>M 15</td>
<td>11</td>
<td>Acute onset of headache, confusion, hallucinations, fever, vomiting.</td>
<td>Increased protein and cell (80 /mm3), OCB -</td>
<td>Thalamic and cerebellar focal hyperintensity; meningeal enhancement</td>
<td>Frontal slow waves</td>
<td>No</td>
<td>NO</td>
<td>NA (3)</td>
</tr>
<tr>
<td>24#</td>
<td>M 64</td>
<td>6</td>
<td>Subacute onset of confusion and apathy; cognitive impairment with memory deficit; hallucination and delusions; delirium</td>
<td>Increased protein and cells (80), OCB -</td>
<td>Normal</td>
<td>Diffuse slow waves</td>
<td>Non Hodgkin lymphoma</td>
<td>Steroids, IVIG, mycophenolate (YES)</td>
<td>3 (12)</td>
</tr>
</tbody>
</table>

Table 4. Clinical data of patients affected with encephalitis
Ataxia

Five patients were finally diagnosed with ataxia. (Table 5) Two women (patient #1 and #14, aged 43 and 26 years respectively) had typical features and both proved to have high serum titers (643289 and 75738 IU/ml). Both had positive oligoclonal bands and showed cerebellar atrophy on brain MRI. They underwent treatment with partial response and achieved only clinical stabilization.

In particular, patient #14 started to develop dysarthria and disequilibrium at the age of 26 years; two years after, T1DM and autoimmune hypothyroidism were diagnosed; she could walk successfully unassisted for some kilometers but in 2016 (18 years after the onset of the disease) she again started to complain of worsening gait and dysarthria. At this time brain MRI revealed severe cerebellar atrophy (figure 7); GAD abs were positive at high titre in serum and CSF; standard examination of CSF was normal, whereas isoelectrofocusing revealed IgG oligoclonal bands.

The patient underwent 5 sessions of PEX without benefit; pernicious anemia was also found and treated with parenteral replacing of B12. Immunoglobulin were also not effective, whereas azathioprine was not tolerated by the patient and then stopped. Eight months later the patient again underwent 5 PEX sessions of plasma-exchange and started weekly maintenance with stabilization of the disease. Before and after three consecutive weekly sessions, GAD abs and total IgG were measured. During each session the titre decreased by about a half and then grew again up to the next session; a parallel removal kinetics of GAD abs and total IgG was observed (Figure 8)
**Figure 8.** Patient 14# GAD abs and total IgG level before and after three consecutive weekly plasma-exchange sessions (arrows)

Both typical ataxic patients were examined by the author; in a late phase of disease, in adjuviction to marked gait ataxia (determining disability with mRS = 4), dysarthria and nystagmus, they showed clear signs of overlapping SPS features, especially rigidity of abdominal, lumbar paravertebral and leg muscles worsening gait difficulty; these symptoms were partially responsive to benzodiazepines and plasma-exchange.

Three women affected by ataxia were considered atypical; two of them have high titer antibodies (229049 and 51475 IU/ml), whereas the third had low titer (39 IU/ml) and was the only patient of this group in which immunotherapy was not performed.

<table>
<thead>
<tr>
<th>N. Pt</th>
<th>Sex; age at onset</th>
<th>GAD serum titer</th>
<th>Clinical features</th>
<th>CSF (IEF)</th>
<th>MRI</th>
<th>Autoimmune comorbidity; tumor; other neural antibody</th>
<th>Immunotherapy (response?)</th>
<th>Last mRS (months follow up)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14#</td>
<td>F 26</td>
<td>643289</td>
<td>Slow onset of dyshartria and balance impairment; abdominal rigidity; depression</td>
<td>Normal (OCB)</td>
<td>Cerebellar atrophy</td>
<td>T1DM; hypothyroidism; autoimmune gastritis.</td>
<td>IVIG, PEX (Yes)</td>
<td>4 (255)</td>
</tr>
<tr>
<td>5#</td>
<td>F 80</td>
<td>229049</td>
<td>Subacute onset of postural instability; vertigo; vomiting; nystagmus and myoclonus</td>
<td>Normal (negative)</td>
<td>Normal</td>
<td>No</td>
<td>Steroids, IVIG (Yes)</td>
<td>3 (12)</td>
</tr>
</tbody>
</table>
30

Table 5. Clinical data of patients affected with cerebellar ataxia (* serum sampled in the chronic phase after immunotherapy)

**Epilepsy**

Only two patients had a final diagnosis of epilepsy (Table 6). Patient #10 was a 60-year-old male with slow onset of temporal lobe epilepsy which was responsive to antiepileptic drugs; he was also affected with thyroiditis. GAD abs were positive at a very high titre whereas standard examination of CSF and IEF were negative. The second patient was a 56-year-old male affected by mental retardation who developed status epilepticus which responded to treatment. No patient underwent immunotherapy; no patient was considered positive for typical features.

Table 6. Clinical data of patients affected with epilepsy

**Other diagnosis**

Only one patient (Patient #4) with a final diagnosis other than the classical spectrum of GAD abs (atypical patients) tested high-titre positive. She was a 45-year-old woman admitted for speech difficulties which had
progressively worsened over three months. T1DM was diagnosed 3 months before the onset of neurological manifestations; at the time of DM1 detection, serum GAD abs were negative. Neurological examination showed spastic dysarthria associated with jaw jerk and brisk reflexes. Mild weakness was observed of the inferior facial muscles. Neither fasciculations nor amyotrophy were documented. Electromyographic recordings from bulbar and spinal muscles did not detect any neurogenic/myogenic potentials while limb motor evoked potentials were normal; no continuous MUP discharges were recorded. Low frequency repetitive stimulation did not detect pathological amplitude decrement. Magnetic resonance imaging studies showed no abnormalities on brain. Anti- ACHR and anti-MUSK were negative but GAD abs were detected at high titre in serum (187202 IU/ml) and CSF (10933 IU/ml). Diazepam was ineffective for the dysarthria. Eight-monthly 5-day courses of intravenous immunoglobulin were administered, followed by azathioprine 3mg/kg/day. A good clinical recovery started after the third infusion, with normalization of the GAD abs. After 16 months, dysarthria was mild and isolated.

A further 8 patients with other final diagnoses had low titre antibodies (14–6547 IU/ml). They were diagnosed with chronic pain (no. 3), Morvan syndrome with positivity for LGI1 abs (n. 1), corticobasal degeneration (no. 1), multifocal neuropathy (no. 1), parkinsonism (no.1), and Wernicke encephalopathy (no. 1). None of these patients with available CSF had GAD abs detectable in the CSF or oligoclonal bands; patient #30 had a mirror pattern of identical IgG bands in serum and CSF. Only one patient had autoimmune comorbidity (patient #26 affected by T1DM). 5/8 patients underwent first-line immunotherapy for a suspected autoimmune disease.

Patient #35 was a 58-year-old male. He slowly developed behavioural changes, gait imbalance, apathy, disinhibition and episodes of delirium. Subsequently he started to develop dysarthria and to be unaware his left arm with contemporary dystonic postures of the left hand. Neuropsychological testing revealed severe apraxia. A corticobasal syndrome was diagnosed; finding serum GAD abs (6547 IU/ml) led to treatment with immunoglobulin with no benefit.

Autoimmune comorbidity
15/35 (43%) patients were affected by co-existent autoimmune diseases. In typical patients autoimmune thyroiditis was highly represented (n. 7) followed by pernicious anemia (n. 3), vitiligo (n. 2), Graves disease (n.1) and psoriasis (n.1). In two atypical patients pernicious anemia, autoimmune thyroiditis and Graves disease were reported. Four patients (2 typical patients) were affected by T1DM.

CSF GAD abs and intrathecal synthesis
A total of 26 patients were tested for GAD abs in the CSF; 11 patients tested positive (range 1248 – 55698 IU/ml). As showed in Table 8, all patients with GAD abs in CSF had high titre GAD abs in the serum. In all 9 cases with availability of paired serum and CSF, intrathecal GAD-ab synthesis was positive. The GAD antibody index (AI) was calculated as reported above.
Nine patients had positive oligoclonal bands (OCB) and 2 patients had an identical pattern of OCB in the serum and in CSF. Notably, in two cases with negative IgG OCB, intrathecal GAD abs antibody synthesis was showed.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Serum titer</th>
<th>CSF titer</th>
<th>AI GAD</th>
<th>IEF pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>6#</td>
<td>SPS</td>
<td>2019681</td>
<td>13032</td>
<td>2,2</td>
<td>Oligoclonal</td>
</tr>
<tr>
<td>8#</td>
<td>Encephalitis</td>
<td>1373559</td>
<td>55698</td>
<td>14,1</td>
<td>Oligoclonal</td>
</tr>
<tr>
<td>9#</td>
<td>SPS</td>
<td>1228265</td>
<td>16432</td>
<td>4,6 **</td>
<td>Oligoclonal</td>
</tr>
<tr>
<td>10#</td>
<td>Epilepsy</td>
<td>1165654</td>
<td>9059</td>
<td>1,9</td>
<td>Negative</td>
</tr>
<tr>
<td>14#</td>
<td>Ataxia</td>
<td>643288</td>
<td>21601</td>
<td>7,2</td>
<td>Oligoclonal</td>
</tr>
<tr>
<td>1#</td>
<td>Ataxia</td>
<td>330016</td>
<td>18388</td>
<td>23,0</td>
<td>Oligoclonal</td>
</tr>
<tr>
<td>5#</td>
<td>Ataxia</td>
<td>229049</td>
<td>3645</td>
<td>6,1</td>
<td>Negative</td>
</tr>
<tr>
<td>4#</td>
<td>Dysarthria</td>
<td>187202</td>
<td>10933</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12#</td>
<td>Encephalitis</td>
<td>99152</td>
<td>2963</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>15#</td>
<td>Ataxia</td>
<td>51475</td>
<td>1248</td>
<td>14</td>
<td>Negative</td>
</tr>
<tr>
<td>2#</td>
<td>Encephalitis</td>
<td>19440</td>
<td>2708</td>
<td>42,1 **</td>
<td>Oligoclonal</td>
</tr>
</tbody>
</table>

*Table 7.* Patients with positive GAD abs in CSF; isoelectrofocusing result and Antibody index. (AI-GAD > 1,5 is suggestive of intrathecal synthesis; ** AI GAD calculated as QGAD/QLim; italic for atypical cases)

*False positives and false negatives*

The aforementioned cut off led to 4 false positives and 3 false negatives.

Two false positive patients (ie atypical patients with high-titre GAD abs) were affected with cerebellar ataxia, one with dysarthria and another with epilepsy.

The ataxic patients recorded as being non-typical had negative findings on standard examination of the CSF and no oligoclonal bands; patient #5 was the oldest woman in the cohort (80 years of age) and had no autoimmune comorbidity; patient #15 did not respond to immunotherapy. Nevertheless, both showed intrathecal synthesis of GAD abs in CSF. The epileptic patient was not resistant to therapy and showed no oligoclonal bands on immunoelectrofocusing; nevertheless he had temporal epilepsy of long duration and autoimmune comorbidity; intrathecal synthesis of GAD abs was found. The patient affected by dysarthria was described above; high-titre GAD abs in CSF were found.

The 3 false negatives (ie typical patients with low-titre GAD abs) were affected by stiff person syndrome spectrum (including two patients with PERM phenotype); one of this patient had serum and CSF glycine receptor antibodies.
Inter-assay and intra-assay precision

One sample was tested 4 times at three different dilutions in the same session to assess intra-assay precision; one sample at four dilutions and other 2 samples were tested in 4 different sessions to assess inter-assay precision. The coefficient of variation (CV) was calculated, yielding percentages within usual accepted values (< 10%) (Table 8,9)

<table>
<thead>
<tr>
<th></th>
<th>GAD abs IU/ml (n.4)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A 1:320</td>
<td>1365,9</td>
<td>9,8%</td>
</tr>
<tr>
<td>Sample A 1:640</td>
<td>578,6</td>
<td>7,3%</td>
</tr>
<tr>
<td>Sample A 1:1280</td>
<td>235,4</td>
<td>7,7%</td>
</tr>
</tbody>
</table>

Table 8. Intra-assay precision (CV = coefficient of variation)

<table>
<thead>
<tr>
<th></th>
<th>GAD abs IU/ml (n.4)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample B (1:80)</td>
<td>256,6</td>
<td>4,9%</td>
</tr>
<tr>
<td>Sample B (1:320)</td>
<td>61,3</td>
<td>6,7%</td>
</tr>
<tr>
<td>Sample B (1:640)</td>
<td>32</td>
<td>7,4%</td>
</tr>
<tr>
<td>Sample B (1:1280)</td>
<td>18,2</td>
<td>7,2%</td>
</tr>
<tr>
<td>Sample C</td>
<td>218,8</td>
<td>3,4%</td>
</tr>
<tr>
<td>Sample C</td>
<td>148,9</td>
<td>10,4%</td>
</tr>
</tbody>
</table>

Table 9. Inter-assay precision (CV = coefficient of variation)

Other neural antibodies

Serum and/or CSF were screened for onconeural antibodies and NsAbs. One patient with a high GAD-abs titre showed co-reactivity with anti-Amphyphisin abs; 4/36 (11%) with low-titre GAD abs had a relevant NsAbs positivity for anti-LGI1 (3) (two affected with encephalitis and patient 21# affected with Morvan syndrome) and anti-GlyR antibodies (1).

Immunoistochemistry and immunoblot

The serum samples of included patients were also screened for GAD abs by IHC (Figure 9) and commercial immunoblot. Positive serum samples showed immunostaining of GABA-ergic nerve terminals on rat cerebellar tissue sections (in particular the granular layer and terminal of basket cells on the Purkinje cell layer).
The results of these tests have been reported in the Table 10 and are displayed together with GAD abs titre (descending order) and intrathecal synthesis of GAD abs. IHC and IB were positive in patients with typical features and high titre antibodies except one SPS patient who tested negative for IB; the lowest value of positivity was 226862 IU/ml (both for IHC and IB). Similar results were obtained with immunofluorescence on commercial kits (Euroimmun). No patient from endocrinological group tested positive, except the patient with the highest titre (405093 IU/ml).

<table>
<thead>
<tr>
<th>Patient</th>
<th>GAD serum</th>
<th>Diagnosis</th>
<th>GAD IS</th>
<th>IHC</th>
<th>IB</th>
</tr>
</thead>
<tbody>
<tr>
<td>6#</td>
<td>2019681</td>
<td>SPS</td>
<td>YES</td>
<td>POS</td>
<td>POS</td>
</tr>
<tr>
<td>8#</td>
<td>1373559</td>
<td>Encephalitis</td>
<td>YES</td>
<td>POS</td>
<td>POS</td>
</tr>
<tr>
<td>9#</td>
<td>1228265</td>
<td>SPS</td>
<td>YES</td>
<td>POS</td>
<td>POS</td>
</tr>
<tr>
<td>10#</td>
<td>1165654</td>
<td>Epilepsy</td>
<td>YES</td>
<td>POS</td>
<td>POS</td>
</tr>
<tr>
<td>14#</td>
<td>643289</td>
<td>Ataxia</td>
<td>YES</td>
<td>POS</td>
<td>/</td>
</tr>
<tr>
<td>13#</td>
<td>514435</td>
<td>SPS</td>
<td>Np</td>
<td>/</td>
<td>NEG</td>
</tr>
<tr>
<td>5#</td>
<td>229049</td>
<td>Ataxia</td>
<td>YES</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>7#</td>
<td>226862</td>
<td>SPS</td>
<td>Np</td>
<td>POS</td>
<td>POS</td>
</tr>
<tr>
<td>4#</td>
<td>187202</td>
<td>Dysartria</td>
<td>Np</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>12#</td>
<td>98841</td>
<td>Encephalitis</td>
<td>Np</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>11#</td>
<td>92028</td>
<td>SPS</td>
<td>Np</td>
<td>/</td>
<td>NEG</td>
</tr>
<tr>
<td>1#</td>
<td>75738</td>
<td>Ataxia</td>
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<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>3#</td>
<td>53993</td>
<td>SPS</td>
<td>Np</td>
<td>NEG</td>
<td>/</td>
</tr>
<tr>
<td>15#</td>
<td>51475</td>
<td>Ataxia</td>
<td>YES</td>
<td>NEG</td>
<td>/</td>
</tr>
<tr>
<td>2#</td>
<td>19440</td>
<td>Encephalitis</td>
<td>YES</td>
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<td>NEG</td>
</tr>
<tr>
<td>35#</td>
<td>6547</td>
<td>CBD</td>
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<td>1182</td>
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<td>/</td>
<td>/</td>
</tr>
<tr>
<td>34#</td>
<td>258</td>
<td>Chronic pain</td>
<td>NO</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>21#</td>
<td>224</td>
<td>Morvan syndrome</td>
<td>Np</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>19#</td>
<td>219</td>
<td>SPS (PERM)</td>
<td>NO</td>
<td>NEG</td>
<td>/</td>
</tr>
</tbody>
</table>
Table 10. GAD abs detected by immunoistochemistry (IHC) and immunoblot (IB);

“/” means that the test was not available

On the basis of clinical assessment, IHC has specificity = 89,47% (CI 66,86 – 98,70), sensitivity = 45,45% (CI 16,75 – 76,62), positive predictive value = 71,43% (36,68% - 91,52%) and negative predictive value = 73,91 % (61,78% - 83,24%).

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29#</td>
<td>169</td>
<td>SPS</td>
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<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>27#</td>
<td>49</td>
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<td>NEG</td>
</tr>
<tr>
<td>32#</td>
<td>41</td>
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<td>NEG</td>
<td>/</td>
</tr>
<tr>
<td>20#</td>
<td>39</td>
<td>SPS (PERM)</td>
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<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>25#</td>
<td>39</td>
<td>Ataxia</td>
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<td>NEG</td>
<td>/</td>
</tr>
<tr>
<td>17#</td>
<td>29</td>
<td>Encephalitis</td>
<td>Np</td>
<td>NEG</td>
<td>/</td>
</tr>
<tr>
<td>28#</td>
<td>27</td>
<td>Encephalitis</td>
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<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>30#</td>
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<td>Parkinsonism</td>
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<td>NEG</td>
<td>/</td>
</tr>
<tr>
<td>18#</td>
<td>21</td>
<td>Wernicke</td>
<td>Np</td>
<td>NEG</td>
<td>/</td>
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<td>23#</td>
<td>18</td>
<td>MMN</td>
<td>Np</td>
<td>NEG</td>
<td>NEG</td>
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<td>31#</td>
<td>14</td>
<td>Chronic pain</td>
<td>NO</td>
<td>NEG</td>
<td>/</td>
</tr>
<tr>
<td>22#</td>
<td>11</td>
<td>Encephalitis</td>
<td>NO</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>33#</td>
<td>11</td>
<td>Epilepsy</td>
<td>NO</td>
<td>NEG</td>
<td>/</td>
</tr>
<tr>
<td>24#</td>
<td>5,4</td>
<td>Encephalitis</td>
<td>NO</td>
<td>NEG</td>
<td>NEG</td>
</tr>
</tbody>
</table>
DISCUSSION

Clinical validation of ELISA

We identified 35 patients with positive serum GAD abs by a modified commercial ELISA. A retrospective clinical analysis, based on data already published in literature, established that 14 cases manifested typical features of GAD neurological autoimmunity described usually in association with high titers.

ROC analysis allowed us to identify a cut off (about 13000 IU/ml) of clinical relevance for serum GAD abs detected by our technique with good accuracy. All control group subjects tested negative, as differently from a previously reported low-titre positivity in 8% of healthy subjects by RIA. [90] The endocrinological control group of patients affected by T1DM and polyglandular autoimmune diseases showed titres lower than 13000 IU/ml in 95% of patients. In our view, these findings are a clinical validation of our method to detect GAD abs in serum and CSF of neurological patients.

Today in most large generalist laboratories where diagnostics of neurological autoimmune diseases are increasingly being performed, [91] GAD abs are routinely detected by commercial ELISA or RIA validated in diabetic patients. These kits identify antibodies at low titres, i.e. the usual range of GAD abs found in T1DM.

Search for GAD abs in neurological patients have not been standardized so far, differently from diabetes. [52] As a consequence, it is difficult to compare values between laboratories due to the heterogeneity of techniques and units of measure. This fact has also been cause for confusion in the literature [49] resulting in various cut offs being used to distinguish high (relevant in neurological patients) from low titers. (Table 11)

<table>
<thead>
<tr>
<th>Paper</th>
<th>Cut off</th>
<th>Disease</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walikonis, 1998 [90]</td>
<td>20 nmol/L</td>
<td>SPS</td>
<td>RIA</td>
</tr>
<tr>
<td>Chang, 2013 [19]</td>
<td>1000 U/ml</td>
<td>SPS</td>
<td>RIA</td>
</tr>
<tr>
<td>Fouka, 2015 [92]</td>
<td>2000 IU/ml</td>
<td>/</td>
<td>ELISA</td>
</tr>
<tr>
<td>Alexopoulos, 2013 [37]</td>
<td>20000 IU/ml</td>
<td>SPS</td>
<td>ELISA</td>
</tr>
<tr>
<td>Vincent 2008 [40]</td>
<td>1000 U/ml</td>
<td>/</td>
<td>RIA</td>
</tr>
</tbody>
</table>

Table 11. Examples of cut offs used to identify high-titre (relevant) GAD antibodies

Looking at literature, we found a large amount of published papers (data not shown) which disregarded the fact that low titers have not a clear role in neurological patients [11], with consequent misleading results and clinical messages. Moreover, a common mistake that is encountered also in clinical practice is to consider a titer over the threshold of the kit (e.g. 2000 IU/ml) as a final value, without performing further dilutions. This may lead to
wrong clinical choices if one considers that the threshold of clinical relevance may be higher and that about 1/5 patients from our endocrinological cohort had values over that cut off. To give an example of such mistakes, the authors of a recent work published by a high impact factor journal failed to show the decrease of GAD abs after plasma-exchange because the titre persisted over the 2000 UI/ml; but they did not obtain an effective titration of the samples before and after the procedure. [66]

Our project aimed to answer these uncertainties and to validate a commercially available kit (designed and validated for endocrinological purposes), optimizing its use in neurological patients, especially to detect antibodies exceeding the kit’s upper threshold (2000 IU/ml) in serum and in CSF. We chose this kit because it is widely available and uses international units of measure. We estimated GAD abs titres of diluted samples by applying a weighted average estimation approach [87] which uses ponderate values based on the distribution in the sigmoid yielded by the experiment. This method provides a better estimation of the titres compared to linear methods (this statement, in our context, is based purely on a theoretical assumption and should be confirmed empirically).

International guidelines recommend, as for onconeural antibodies for which commercial tests are widely available, [93] [94] to screen neurological patients for GAD abs using a two-step method i.e. immunohistochemistry (IHC) on rat cerebellum followed by confirmation by another technique, as RIA, ELISA [49] or immunoblot. It has been already observed that IHC is very specific in detecting relevant GAD abs in neurological patients (we confirmed this observation, as IHC got specificity = 89%); on the other hand, it is positive only in very high titre neurological patients (e.g. over 2000 U/ml [11] or 4000 U/ml [19] by different commercial RIA kits; note should be taken that the unit of measure does not convert linearly to IU/ml). Notably, this criterion is based on one assumption that has not been rigorously validated in a clinical setting to our knowledge. Moreover, IHC is often not available in generalist laboratories and interpretation of morphological patterns requires high expertise, which is only available in a limited number of reference centres. Our work highlights that also patients with intermediate titres (13000 – 200000 IU/ml) could have relevant GAD abs – with a clinical profile similar to patients with higher titers - but testing negative to IHC, which achieved a sensitivity of 45%. This low sensitivity may be due to differences in the preparation of tissues described in similar works [11] [50], although our protocol is comparable to highly sensitive IHC performed for the detection of neuronal surface antibodies [95]

It has been recommended [11] that in the presence of serum positivity for GAD abs, the relevance of these findings (to justify, for example, an immunosuppressive treatment) should be based on clinical findings and confirmation of intrathecal synthesis, which is very frequent in GAD abs associated diseases. [27] [60] From this standpoint, the clinical relevance of our threshold was confirmed by the fact that intrathecal synthesis of GAD abs (the observer was blinded to this finding) was only found in patients with high titer (Table 7). Sunwoo in 2016 [96] reported a series of patients with GAD abs CSF positivity without detection of antibodies in paired serum. In that study GAD abs were detected by RIA at very low titres and in a good number of cases patients
had a final diagnosis of neurodegenerative disorders. In our experience, since 2009, we have found only one positivity on CSF with sero-negativity in a patient affected by multiple sclerosis. Notably in our cohort, intrathecal synthesis of GAD abs was found also in two cases with negative IgG oligoclonal bands; this is in line with previous observations [39] [60] and with the notion that Antibody Index evaluation is more sensitive than oligoclonal band detection by immunoelectrofocusing. [97]

We confirmed that caution must be taken to handle results from diabetic patients: in two cases we demonstrated very high titers in endocrinological group control; this point has been already underlined in one study showing that patients affected by T1DM and high-titre GAD abs during a long follow up did not develop neurological disturbances. [71]

**Clinical features of patients with typical GAD spectrum**

The clinical features of our high-titre patients are similar to the ones already reported in literature. Stiff-person syndrome is the most widely-recognized disease associated with GAD abs. [11] With respect to other series of GAD abs positive patients, [11] [16] in our work there is an over-representation of patients affected by encephalitis (10 cases, 3 typical with a high titre) compared to ataxia (5 cases, 2 typical with high titre). This is probably due to a selection bias: our laboratory accepts samples from the whole of Italy for the detection of recently described surface neuronal antibodies, particularly in patients affected by encephalitis [98]. In our cohort we did not identify patients with clinical manifestation reported more rarely in literature, as myasthenia gravis [99] and myelopathy [100].

The diagnoses of SPS were all confirmed in our GAD positive patients by clinical retrospective review. SPS has specific manifestations and diagnosis is based on clinical and electrophysiological data consistent with spinal hyperexcitability [86]. GAD abs are a supportive criterion for the diagnosis, considering that up to 20% of patients with a diagnosis within the SPS spectrum can be seronegative. This subgroup of patients more often has a limited form of SPS or PERM and can be paraneoplastic in a higher proportion of cases; moreover, they show a lower incidence of autoimmune comorbidity. [53] [101] Our 3 patients with low GAD abs had no associated autoimmune diseases (compared with all the high-titre patients) and two cases had a PERM phenotype. Notably, one patient affected by PERM was positive for Glycine receptor (GlyR) antibodies in serum and CSF. These antibodies have recently been described in association with PERM, sometimes in co-existence with GAD abs. Moreover, one study recently found GlyR abs in 15% of patients affected by classical SPS. [37] We cannot exclude that these low titres of GAD abs may be of relevance, but the different clinical profiles in patients with PERM suggest involvement of other antibodies.

A total of 5 women in our cohort were diagnosed with cerebellar ataxia. Only two patients were considered to have typical ataxia on the basis of criteria explained in the methods section. The over-representation of women has been confirmed in the most significant series published about this phenotype. [23] [71] Clinical features consisting of gait ataxia, diplopia and nystagmus were present; both typical patients in our cohort had a slow
onset but subacute onset was also present. We performed repeated cycles of plasma-exchange, obtaining stabilization of the disease, in a woman whose conditions had started worsening 18 years after the onset of cerebellar ataxia. We showed that the GAD-antibody titre decreased after each session, together with total IgG. In the following days both the GAD-antibody titre and total IgG progressively increased to near pre-treatment values. These findings confirm that plasma-exchange is effective in decreasing both the GAD-antibody titre and total IgG. The reduction of the GAD antibodies burden by plasma-exchange has previously been shown in GAD-related diseases although, globally, the response to this therapy is partial and transitory.

A further interesting observation in both our high-titre ataxic patients was the co-existence of rigidity and spasms of the lumbar and abdominal muscles and legs in a late phase of the disease. The coexistence of typical symptoms of SPS in patients with GAD-antibody-related ataxia has already been reported in 26% of patients in a single series. These symptoms have already been described after the development of ataxic syndrome but in some cases they precede the onset of cerebellar ataxia by a few years, resembling the focal form of SPS.

This finding highlights the notion, which has been clear since the earliest description of stiff person syndrome, that there is an overlap of clinical manifestations related to GAD antibodies. A further such example is a patient from our series, who developed a clinical picture of limbic encephalitis (according to the recent clinical diagnostic criteria), complicating the picture of a patient affected by SPS. In the series by Malter, SLS was diagnosed during the course of limbic encephalitis in 2 GAD-antibody positive patients. In another case, myoclonus and facial cramps were reported during GAD-antibody-related encephalitis.

Clinical features of our patients affected by encephalitis with high titre antibodies, showing clinical, radiological and neurophysiological involvement of the mesio-temporal lobes, confirm that this reactivity must be considered in the screening of patients affected by limbic encephalitis. The low number of patients with high-titre abs did not allow to detect some specific clinical finding that could differentiate this form from encephalitis associated to neuronal surface or onconeural antibodies. We confirmed a bad outcome in spite of immunotherapy, especially for residual memory deficits, similarly to paraneoplastic limbic encephalitis. Most patients affected with encephalitis testing low-titre positive had non-specific features and they did not resemble the clinical picture of limbic encephalitis. Nevertheless, a good response to immunotherapy was reported; notably 3 low-titre patients tested positive for LGI1 abs. One patient had a clinical onset dominated by facio-brachial dystonic seizures, that typically associate to LGI1 encephalitis; she was promptly treated with excellent outcome. Another LGI1 positive patient showed more aspecific findings with multifocal encephalopathic involvement, whereas the third was a patient diagnosed with Morvan syndrome.

Epileptic patients harbouring serum GAD antibodies have been variably considered in the literature. In our view the correct approach to take is to consider only high titres of GAD abs as being significant. Nevertheless, there are no convincing data describing the clinical features of epileptic patients testing positive for GAD abs. The most consistent studies reported temporal epilepsy with frequent drug-resistance and long duration.
Due to this uncertainty, our patients were considered atypical, although one patient showed high-titre GAD abs (false positive). Observational studies about this specific group of patients could address this issue in the future.

**Atypical cases**

In our cohort we identified the unusual case of a 45-year-old Caucasian woman developing spastic dysarthria, jaw jerk and brisk reflexes without cerebellar manifestations; mild weakness of the inferior facial muscles was observed; these features were associated with the detection of high-titre GAD abs in the serum and CSF and the co-existence of T1DM. The patient clearly responded to intravenous immunoglobulin. This case could resemble prominent brainstem involvement in some GAD-abs-positive cases already reported by Pittock [100]; in particular 4/11 African patients in this series had dysarthria (in some cases described as spastic) in the context of more complex brainstem involvement. This case could probably been interpreted as a manifestation of focal dysfunction of inhibitory truncal circuits, leading to focal rigidity in absence of the truncal or limb rigidity typical of SPS. We also described a 58-year-old male that slowly developed a corticobasal syndrome; finding of GAD antibodies on serum (6547 IU/ml) led to treatment with immunoglobulin with no benefit. Oligoclonal bands and GAD abs in CSF were negative. In 2014 two patients with high-titre GAD abs positive SPS who have signs suggestive of corticobasal syndrome have been described; they had positive oligoclonal bands in CSF. [114]

These observations highlight that GAD autoimmunity could express with signs resembling clinical phenotypes in which an inflammatory pathogenesis could be overlooked.

**Autoimmune and paraneoplastic accompaniments**

No tumor accompaniment was detected in our series; this finding confirms that GAD autoimmunity in most cases is not associated with a systemic cancer and that careful oncological screening should be limited to GAD abs in the context of rare classical paraneoplastic neurological syndrome. [82][94]

We confirmed a high association of GAD abs with coexisting autoimmune diseases, especially autoimmune thyroid disease and T1DM; [20] this is not a common finding of other antibody-associated neurological syndromes (like paraneoplastic disease or autoimmune encephalitis), except for neuromyelitis optica in which the most frequent accompaniments are Sjogren syndrome and lupus erythematosus. [115]

**Coexistence with other neural antibodies**

Several studies have addressed this issue, in order to establish if autoimmune diseases associated with GAD abs could be due to neuronal surface antibodies with higher pathogenic role. [16] [19] [37] [116]

In our patients with high-titre GAD abs no other known antibody was detected, except one co-reactivity with Amphyphisin in one epileptic patient. These onconeural antibodies are usually found in paraneoplastic SPS [100]. A very long follow-up for this patient is available (97 months) and no tumor have been detected.
Three patients with low-titre GAD abs were found positive for LGI1 abs and one with GlyR antibodies. We did not search for anti-gephyrin and GABARAP, which had previously been suggested as possibly having co-reactivity with GAD abs. [19]. Whether the coexistence of neuronal surface antibodies with low-titer GAD abs is incidental or due to immunologic mechanisms (eg. cross reaction) is difficult to establish (see the next section).

Meaning of low-titer GAD abs

One of our aims was to explore the meaning of low-titre GAD abs, on the basis of our identified threshold. We identified 20 patients antibodies under the threshold of 13000 (6 – 6547 IU/ml). Of them, 3/20 (15%) had typical GAD abs manifestations (false negatives). 17 patients (true negatives) had atypical features. They were prevalently male (10/17; 59%) with a mean age of 49 years. No patient had intrathecal synthesis of GAD abs. 2/11 (18%) patients had IgG oligoclonal pattern in CSF. 3/17 (18%) had autoimmune comorbidity. 7/20 (35%) had a final diagnosis that excluded any autoimmune causes (neurodegenerative or metabolic disorders). However, considering that patients showed a partial response to immunotherapy in 9/12 cases (75%) and that in 4 cases relevant co-existent antibodies were detected, our data suggest that low titres don not rule out the possibility of an underlying autoimmune mechanism, on which grounds, in case of clinical suspicion, a complete neuronal autoimmune panel has to be performed. Diagnostic value of low-titre GAD abs is still debated; recently some authors have claimed a relevance in some clinical phenotypes, [117] [118] but these data need further confirmation because casual association has not been excluded undoubtedly.

Conclusion

We are aware of the limits of our work, based on retrospective and indirect clinical information, and take due caution in proposing a cut off that depends on our data (GAD abs titre is not a normally distributed variable).

Managing GAD- abs positivity in patients with suspected autoimmune neurological diseases is a very common problem in clinical neuroimmunology. Our work provides some suggestions on how to consider the values yielded by this specific commercial kit. When addressing GAD positivity in clinical practice, we confirm the well-known recommendation to also screen CSF and calculate intrathecal synthesis. We additionally suggest to obtain an accurate titration of serum (if possible with standardized units of measure) in order to better consider the clinical relevance of GAD abs and to provide the right diagnostic and therapeutic choices for our patients.

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