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AUTOANTIBODIES AGAINST NEUROGLIAL SURFACE ANTIGENS: LABORATORY STRATEGIES FOR THEIR DETECTION AND ROLES IN ACQUIRED DEMYELINATING SYNDROMES, AUTOIMMUNE ENCEPHALITIS AND ACQUIRED NEUROMYOTONIA

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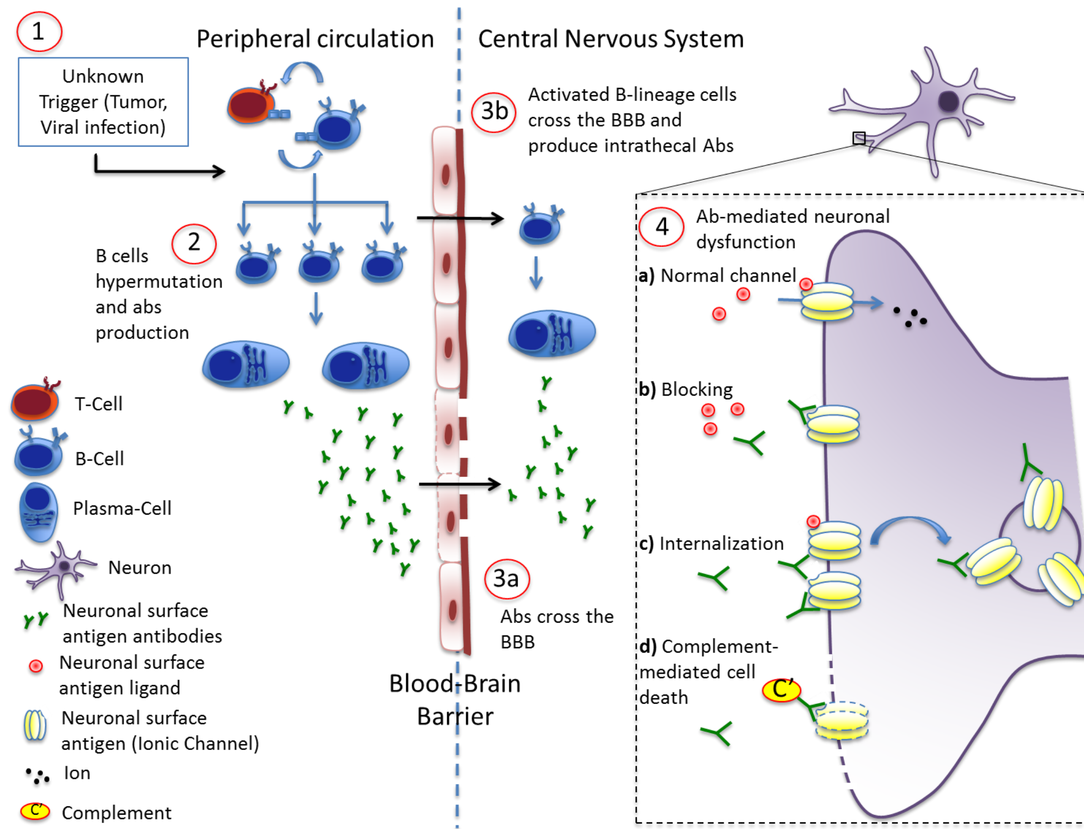
1 Introduction

1.1 Neuroglial surface antibodies in neuroimmunology

In the past years, the neuroimmunology field has been revitalized by the identification of antibodies targeting surface proteins of neurons or glial cells (neuroglial surface antibodies).[1] These autoantibodies are considered useful for two main reasons: a) they are very specific biomarkers of the associated neurological disorders; b) they are likely to be pathogenically relevant for the neurological disorder. This is crucial for the immediate implications in clinical practice: patients with neuroglial surface antibodies mediated disorders can be treated effectively with procedures able to remove the autoantibodies from the peripheral circulation or to suppress their production. The autoantibodies can cause surface protein dysfunction through different mechanisms, such as cross-linking and internalization, complement mediated cell destruction and functional block (Figure 1). The latter mechanism is more common with IgG4 autoantibodies, such as LGI1 in limbic encephalitis and NF-155 in chronic demyelinating polyradicoloneuropathy.[2]

Figure 1: potential pathogenic mechanisms of neuronal surface antibodies (from *Gastaldi et al, 2016*)[3]



- The pathogenesis of disorders associated with neuronal surface antibodies is still unclear. Several potential triggers have been proposed as the first determinant of an aberrant activation of the immune system (1). In NMDAR encephalitis it is well recognized that a tumor (mainly an ovarian teratoma) or an herpetic infection can precede the onset of the disease, but in the majority of cases the trigger remains unknown. In the peripheral circulation B lymphocytes, after interaction with T-helper lymphocytes, become activated and undergo somatic hypermutation and differentiation, starting the autoantibody production (2). Antibodies against neuronal surface antigen may subsequently reach the central nervous system by crossing the blood brain barrier at sites of increased permeability (3a). It is also likely that activated B-lineage cells are able to actively cross the blood brain barrier and undergo the same differentiation process within the central nervous system, contributing to the intrathecal pool of autoantibodies (3b). When the antibodies reach their target, the normal function of the surface antigen (usually a ionic channel, 4a) can be altered by different mechanisms. The antibodies may prevent the binding of the channel ligand (blocking, 4b); some antibodies cause cross-linking and internalization of receptors and thus depletion from the cell surface (4c); finally, antibodies may activate the complement cascade and induce neuronal death (4d).

The gap between the definition of an autoantibody antigenic target and the demonstration of its pathogenicity has been and still is extremely challenging. In 1957, Witebsky et al. proposed postulates to establish the autoimmune etiology of a disease, that were later revised in 1991 by Bona and colleagues.(Table 1)[4,5] These postulates include both direct and indirect proofs that link the antibody to a specific disease, and for many of the novel neuroglial surface antibodies, the postulates are far from being completely fulfilled. A paradigmatic example of this issue concerns MOG antibodies, that will be extensively treated throughout this thesis. Despite MOG has been identified as a potential antibody target in demyelinating disorders for several years, and the autoantibodies have been identified in large cohorts of patients with demyelinating clinical phenotypes, evidence supporting their pathogenicity in animal models is still scarce. On the other hand, MOG peptides are currently used to induce experimental autoimmune encephalitis (EAE), an animal model of multiple sclerosis and acute disseminated encephalomyelitis.[6]

Currently, several issues are still open in autoantibody-mediated disorders of the central nervous system (CNS). First of all, the mechanisms that lead to the production of antibodies against brain self-antigens are still obscure. It is possible that immunological responses against foreign microorganisms with shared epitopes with self-antigens could lead to autoimmunity in some cases (molecular mimicry); alternatively auto-reactive B- and T-cells could develop following clinical or subclinical brain injury, with the exposure of myelin proteins and neuronal antigens. Secondly, peripherally produced autoantibodies, in order to be pathogenic, the autoantibodies need to reach their target within the CNS, possibly crossing the blood brain barrier in areas of altered permeability. Alternatively, activated B- and T-cells, once activated in lymphoid organs, could cross an intact blood brain barrier and initiate an intrathecal production of antibodies, as it has been hypothesized for NMDAR encephalitis.[7]

From a practical point of view, the high clinical relevance of these autoantibodies for diagnostic purposes demands high accuracy in their laboratory detection. Since their discovery, it became soon clear that classic immunoassays, which imply complete denaturation (western blot), or at least partial alteration of the antigen (ELISA) were not suitable for the identification of neuroglial surface antibodies. Indeed, these antibodies recognize discontinuous epitopes that are exposed only when the tertiary structure of the protein is preserved. The introduction of novel

detection techniques on one hand has increased the identification of neuroglial surface antibody associated disorders (see the following section), and on the other hand has helped to define the associated clinical phenotypes. In this field, however, standardization of the laboratory procedure is essential for the sake of accuracy, but far from being achieved.

In this thesis we will provide results from the implementation of several techniques for the identification of neuroglial surface antibodies at the Pavia Neuroimmunology laboratory. We investigated the strategies currently used by most laboratories worldwide and we tried to compare them in order to identify the most efficient ones. We then exploited such strategies to dissect the clinical correlates of neuroglial surface antibody associated disorders, and to identify novel antigen targets in this field.

Table 1: Evidence of autoimmunity in antibody-mediated disorders of the nervous system (Witebsky's postulates revisited)[4,5]

			Disease			
			Myasthenia Gravis	NMO	NMDAR encephalitis	MOGAD
Evidence		Explanation	Autoantibody			
			AchR	AQP4	NMDAR	MOG
Direct proof	Direct transfer of antibodies reproduces the disease	• Autoantibodies from patients are able to transfer the disease in animal models • Transplancentar transfer of autoantibodies reproduces the disease in fetus • Autoantibodies can induce the destruction of cells expressing the antigen in-vitro	+++ (Maternal and animals)	+++ (Animals)	++ (Maternal and animals)	+/- (Animals)
Indirect proof	Reproduction of autoimmune disease in experimental models and/or isolation of autoantibodies	• Experimental immunization of animal models using the autoantigen can reproduce the disease • Autoantibodies can be eluted from the target organ or found in lesions	+++	-	+ (pathology from patient's brain)	+ (pathology from patient's brain)
Circumstantial evidence	Clinical clues from human disease suggest an autoimmune disease	• Association with other autoimmune diseases • Lymphocytic infiltration of the target organ • Statistical association with a particular MHC haplotype • Favorable response to immunosuppression	+++	+++	++	++

The table describes Witebsky's postulates adapted from Bona et al. in 4 antibody-mediated disorders of the nervous system. NMO: neuromyelitis optica; MOGAD: MOG associated disease; MHC: major histocompatibility complex.

1.2 Laboratory techniques for neuroglial surface antibodies detection

As previously mentioned, neuroglial surface antibodies are conformational and recognize discontinuous epitopes that strictly depend on the tertiary structure of proteins. The issue of detecting conformational autoantibodies with sufficient sensitivity has been solved only recently, and the breakthrough in this field has been achieved by the development of cell-based assay (CBA) technology.[1] CBAs rely on immortalized cell lines that are presumed to share very few antigens with neurons and that are easily cultivable *in vitro*, such as the human embryonic kidney-derived (HEK293) cells. These cell lines are transfected with a cDNA plasmid coding for the protein of interest, allowing its over-expression. (Figure 2) In the case of transmembrane proteins, the insertion of the target protein into the lipid bilayer of cell membranes ensures the preservation of the native structure, thus allowing the binding by conformational antibodies and its detection with indirect immunofluorescence. An additional advantage is that CBAs can be devised using live cells (LCBA), that prevent the autoantibody access to intracellular epitopes (less likely to be relevant *in vivo*), and that completely preserves the conformational nature of binding sites. LCBAs can only be performed in a laboratory with cell culture facilities, and are necessarily home-made tests. Fixed CBAs (FCBA), on the other hand, allow increased durability of the substrate and can be used in commercially available kits. It must be considered, though, that the procedure of fixation could, in principle at least, alter the conformational state of the extracellular epitopes, and expose non-relevant nuclear or cytoplasmic antigens recognized by other autoantibodies (e.g.: anti-nuclear antibodies) resulting in higher background. Overall, these factors could make FCBAs less sensitive and accurate than LCBAs.

Figure 2: live and fixed cell based assay for the detection of neuroglial surface antibodies (from *Gastaldi et al, 2016*)[3]

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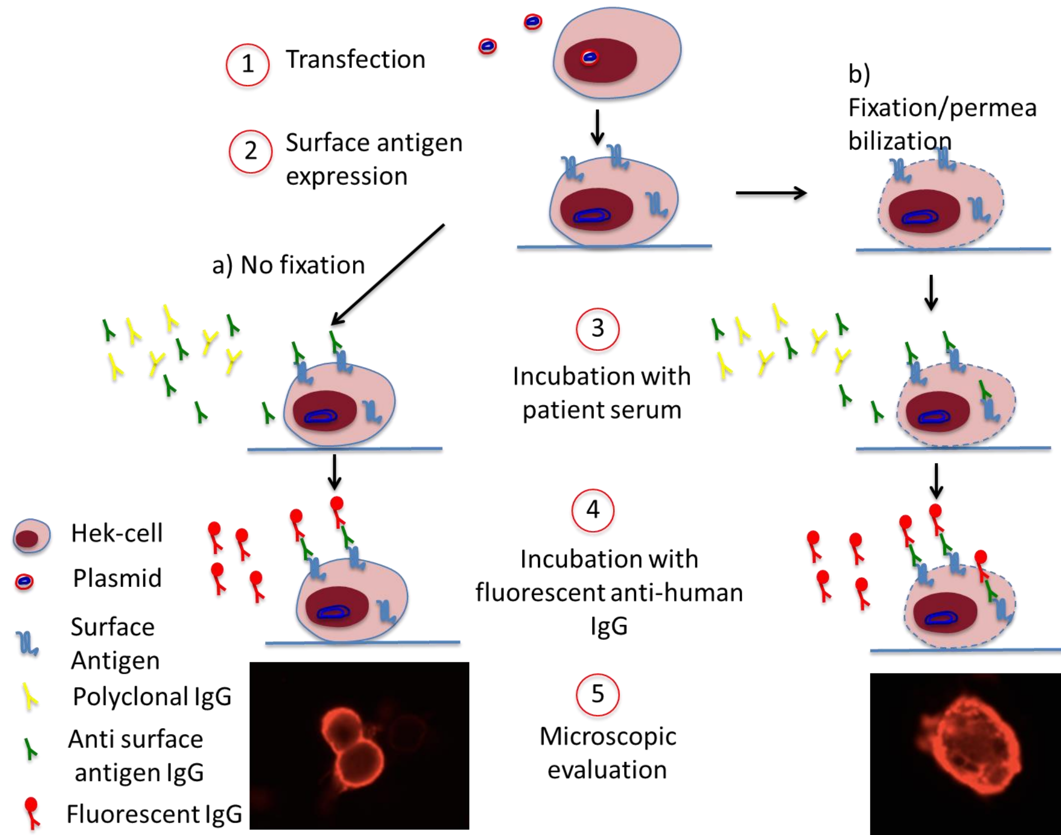


The cell-based-assay (CBA) is a technique that allows identification of antibodies whilst preserving the tertiary structure of the antigen. Live HEK293 cells are transfected using plasmids that contain DNA coding for the antigenic target (1); transfected cells express the antigen mainly (but not exclusively) on their surface (2); cells can either be stained live (a), or be fixed and permeabilized (b), and are subsequently incubated with patient serum or cerebrospinal fluid (3). Specific antibodies in the serum or CSF will bind the expressed antigenic target; note that when cells are alive specific antibodies are able to bind only antigenic targets expressed on the cell surface (a), whilst when cells are fixed/permeabilised intracellular antigens can be reached (b). Cells are then incubated with a secondary fluorescent antibody that recognizes human IgG (4) and the presence of fluorescent anti-human IgG is detected with a fluorescent microscope (5).

In addition to CBAs, immunohistochemistry on murine brain (IHC) represents another cornerstone technique for the detection of conformational autoantibodies. This assay is not a novelty in neuroimmunology and has been used to identify most of the onconeural antibodies. Indeed, a double step technique combining staining on primate cerebellum followed by a confirmatory line blot is nowadays the mainstay for

the detection of onconeural antibodies,[8] and for this purpose, the animal brain is intensely fixed through perfusion and permeabilized, in order to facilitate the access of the autoantibodies to their intracellular target. To identify neuroglial surface antibodies however, it must be considered that the conformational state of the proteins can be compromised by prolonged fixation, and light fixation procedures are mandatory to preserve the discontinuous epitopes (see methods section).[9] In brain slices treated with light fixation protocols, neuroglial surface antibodies can be identified through their binding to specific brain areas. The biggest advantage of IHC is that it displays a wide range of antigens in a single test, allowing the identification of a large number of neuroglial surface antibodies. Through the interpretation of the staining pattern it is possible to guess the specificity of the antibody. For example, antigens such as NMDAR or CASPR2 are located in areas defined as “neuropilum”, with poor representation of cell bodies, but high density of synapsis. NMDAR antibodies provide a characteristic staining located specifically on the molecular layer of the hippocampus, whilst CASPR2 antibodies provide a more diffuse staining involving hippocampus, brain cortex, basal ganglia and cerebellum (see figure 3 and figure 27 at page 111). A detailed description of the staining patter according to the antibody specificity is reported in table 2. In addition, IHC is able to identify neuroglial surface antibodies directed to antigens yet to be characterized, and has played a crucial role in the identification of several antibodies including NMDAR, $GABA_AR$ and DPPX. IHC has also some limitations. First of all, the light fixation procedure implicates a higher level of background and non-specific nuclear and cytoplasmic staining in neurons and glial cells, especially using serum samples. For this reason, such reactivities should be evaluated carefully. Secondly, due to similarities between staining patterns, this technique is by definition non-antigen specific, and always requires a confirmatory test with an antigen-specific assay such as the CBA. Finally, the identification of an uncharacterized neuropilar staining is only suggestive, but non demonstrative of a neuroglial surface antibody. Indeed, the rat brain slices used to perform the staining are 7-12 micron thick, offering a certain degree of intracellular antigen exposure despite the absence of permeabilizing detergents throughout the procedure (see methods section, page 26).

Figure 3: Staining patterns of neuroglial antibodies on rat brain immunohistochemistry

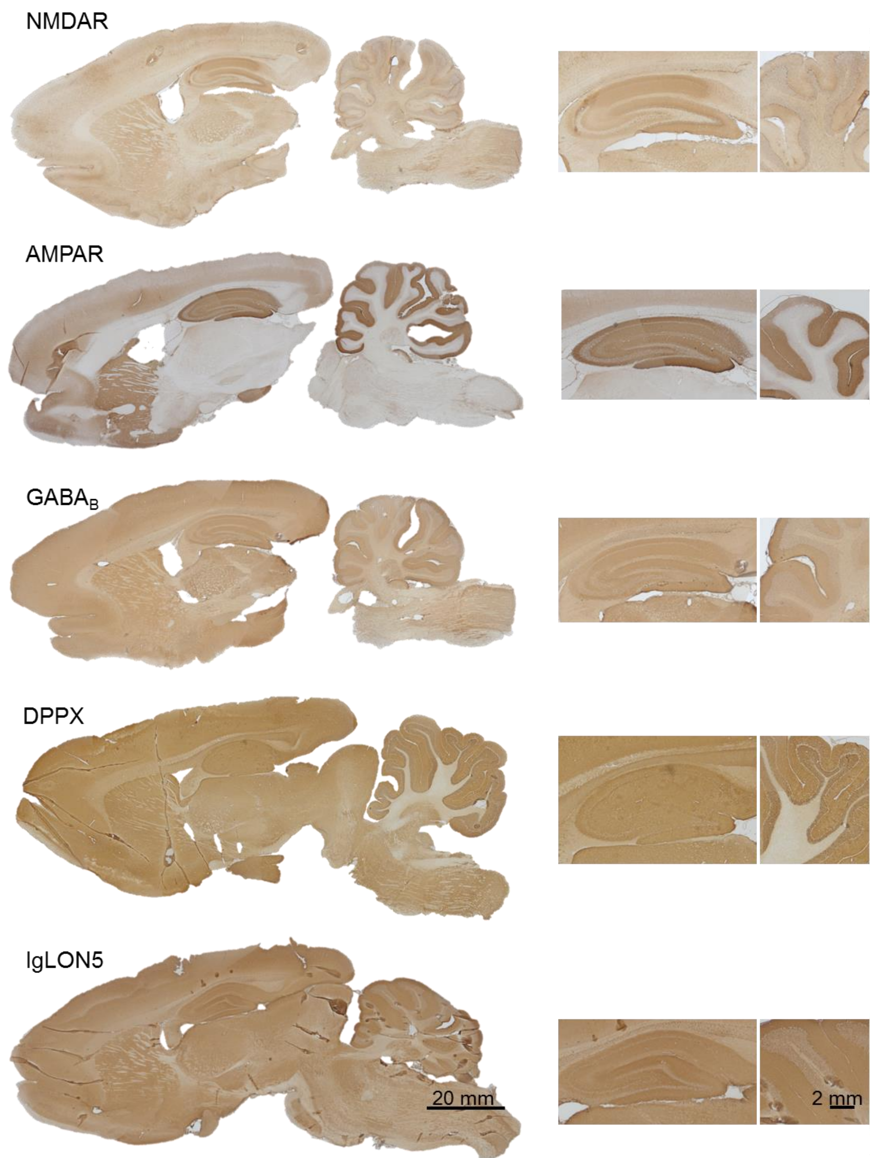
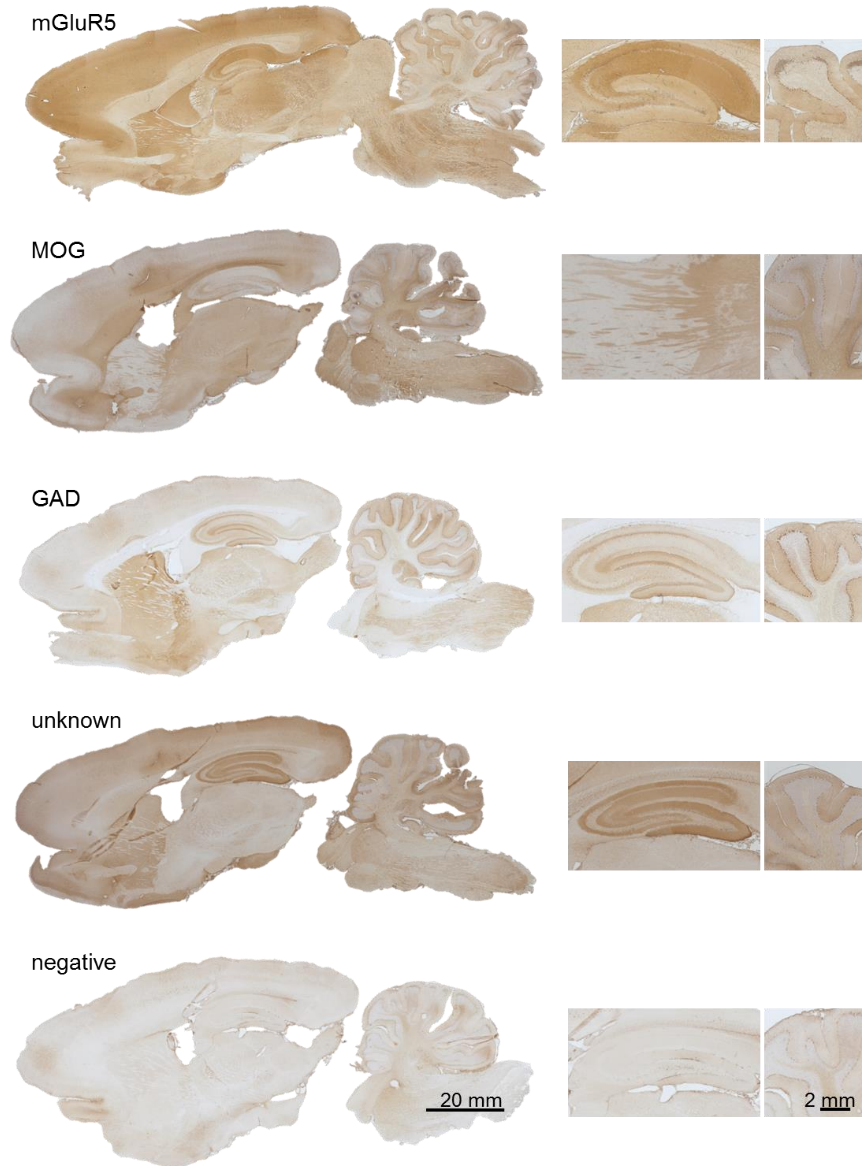


Figure 3: Staining patterns of neuroglial antibodies on rat brain immunohistochemistry (continues)



The figure represents typical staining pattern for neuronal surface antigens (NMDAR, AMPAR, $GABA_B$, DPPX, IgLON5, mGluR5), a glial surface antigen (MOG) and a synaptic intracellular antigen (GAD), compared to a negative control. A sample providing an uncharacterized hippocampal neuropilar staining is shown (labelled as "unknown"). Starting from left: whole brain sagittal section, detail on hippocampus, detail on cerebellum. For MOG antibodies basal ganglia are shown instead of the hippocampus, to better appreciate the white matter fibers stained within the striatum. Staining patterns for LGI1 and CASPR2 are not shown, and can be found in figure 27 at page 111. Immunohistochemistry on rat brain, patient IgG are revealed using DAB (brown color). Scale bar is at the bottom of each page.

Table 2: rat brain immunohistochemistry staining pattern for neuroglial antibodies

ANTIGEN	HC	HC pattern	CEREB	CEREB pattern	CORTEX	BASAL GANGLIA	WHITE MATTER	VESSELS	Comments
NMDAR	+++	DG molec +++(line adjacent to pyramidal cell layer more intense) CA molec ++ Subiculum - Hilus -	NEG	-	NEG	NEG	NEG	NEG	Might show some faint staining in granular layer of Cereb., thalamus, striatum
LGI1	+++	DG molec +++(2/3 outer layer more intense, evidence of a "line" that separates the molec layer) CA molec +++ Subiculum +++ Hilus +++	+++	molec layer +++ ("comb-like"fibres are visible at the edge of molec/gran penetrating in the granular) granular -	++	++	NEG	NEG	-
CASPR2	+++	DG molec ++ CA molec +++(more intense in CA1-2) Subiculum++ Hilus ++	+++	Molec+++ Granular+++	++	++	NEG	NEG	The increased staining in CA1-2 could be absent
GABAB	+++	DG molec ++ CA molec +++ Subiculum++ Hilus ++	+++	Molec+++ Granular+++	++	++	NEG	NEG	Low titres can provide only a HC staining
GABAA	+++	DG molec ++ CA molec +++ Subiculum++ Hilus ++	+++	Molec+++ Granular+++	++	++	NEG	NEG	Low titres can provide only a HC staining
AMPAR	+++	DG molec +++ CA molec ++ Subiculum++ Hilus +(faint reticular)	+++	Molec+++ Granular-	++	+++striatum	NEG	NEG	-
NEUREXIN-3A	+++	DG molec ++ (inner 1/3 stronger, forms a "line" in the molec layer) CA molec +++ Subiculum+++ Hilus +	+++	Molec+++ Granular++	+++	+++	NEG	NEG	-

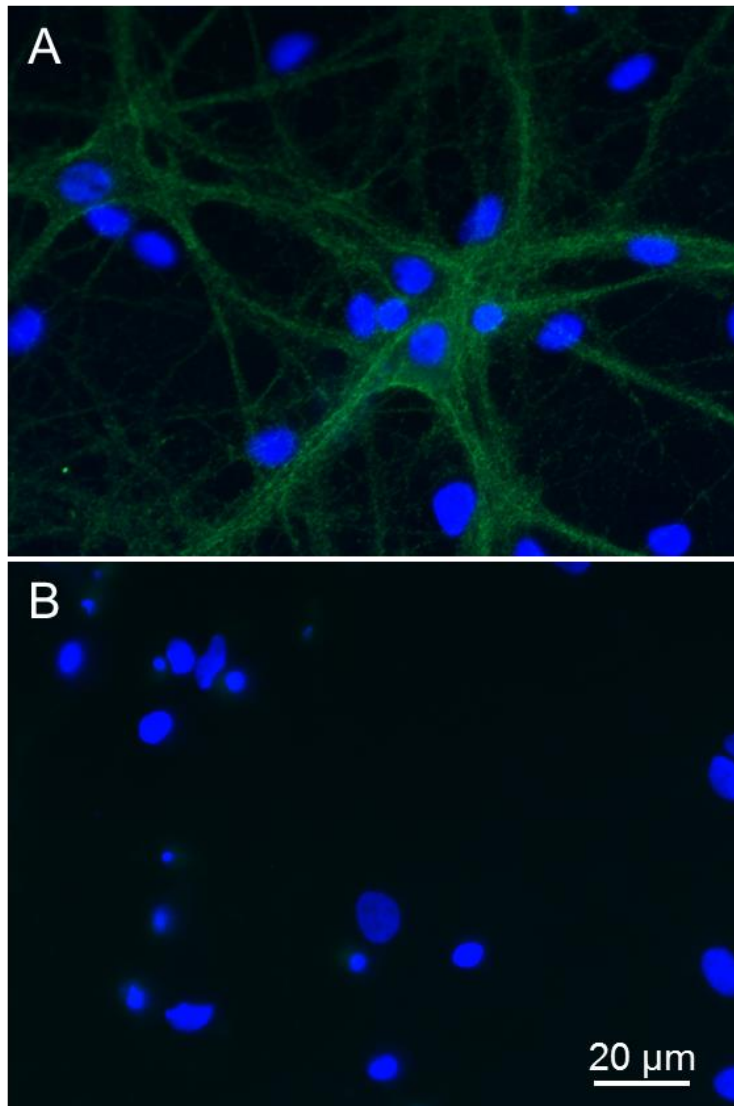
Table 2: rat brain immunohistochemistry staining pattern for neuroglial antibodies (continues)

ANTIGEN	HC	HC pattern	CEREB	CEREB pattern	CORTEX	BASAL GANGLIA	WHITE MATTER	VESSELS	Comments
IgLON5	++	DG molec +++ CA molec +++ Subiculum+++ Hilus +++	+++	Molec+++ Granular+++	+++	+++	NEG	NEG	-
Amphyphysin	+++	DG molec ++ CA molec +++ Subiculum++ Hilus +++	+++	Molec+++ Granular+++	+++	+++	NEG	NEG	-
GAD	+++	DG and CA molec (+) "Basket cells" around the pyramidal cell layer +++	++	"Basket cells" around the granular layer cells+++ Matrix of cereb deep nuclei+++	++	++	NEG	NEG	-
DPPX	+++	DG molec +++ CA molec +++ Subiculum+++ Hilus +++	+++	Molec+++ Granular+++	+++	+++	NEG	NEG	The appearance of the staining is "granular"
DNER/Tr	NEG	NEG	+++	Molec+++ Granular-	NEG	NEG	NEG	NEG	-
mGlur1	+	DG molec + CA molec + Subiculum- Hilus -	+++	Molec+++ Granular-	NEG	NEG	NEG	NEG	-
mGlur5	+++	DG molec++ CA molec+++(more intense in CA1 and 2) Subiculum++	NEG	-	++	+++(striatum)	NEG	NEG	The increased staining in CA1-2 could be absent or extremely evident so that the DG is almost negative
AQP4	+	DG molecular + Subgranular zone++ Hilus+ SLM++	+++	Granular(basket staining around granular cells and Purkinje)+++	NEG	NEG	++	+++	-
MOG	NEG	NEG	NEG	NEG	NEG	NEG	+++	NEG	-

HC: hippocampus; CEREB: cerebellum; DG: dentate gyrus; CA: cornus ammonis; molec: molecular layer; SLM: stratum lacunosum moleculare

Another useful technique in neuroglial surface antibodies detection is represented by the primary hippocampal neuronal cultures. Hippocampi are extracted from embryonic rats and dissociated into neurons and then cultured *in-vitro*, where they are able to form mature synapses. The live neurons provide the substrate for antibody binding, and the integrity of the cell membrane guarantees that the antibody-antigen interaction is occurring at the neuronal surface.(Figure 4) Neuronal cultures, although technically challenging and time consuming, can be useful to confirm the surface location of the antigen for antibodies identified with neuropilar staining on IHC. This indicates a higher probability for the antibody to be able to interact with the target antigen in-vivo, and therefore be pathogenic and clinically relevant. In addition, neuronal cultures provide a valid substrate for precipitation experiments that, followed by mass spectrometry analysis, can help to identify novel neuroglial surface antibodies (see page 28).

Figure 4: Binding of neuronal surface antibodies to live neuronal cultures



The figure shows the binding of the serum from a patient with IgLON5 IgG antibodies to live rat hippocampal neurons cultured in vitro (A, green). No staining is detected using a serum from a healthy control (B). Blue: DAPI, which stains nuclei. Scale bar is in the bottom right corner.

2 Neuronal surface antibodies and autoimmune encephalitis

2.1 General Introduction

2.1.1 Neuronal antibodies: overview

Autoantibodies targeting neuronal proteins have been known for several years in neuroimmunology, and were firstly described in patients with CNS or peripheral nervous system (PNS) disorders (e.g. limbic encephalitis or sensory ganglionopathy) associated with the presence of an underlying tumor, and were therefore defined "onconeural".[10] Such antibodies target intracellular neuronal proteins (intracellular neuronal antibodies, IC-NAbs) that are concomitantly expressed by the tumor (e.g. Hu, Yo, Ma2), and are part of the physiologic response of the immune system against the tumor itself. In paraneoplastic disorders, IC-NAbs are not the primary cause of neuronal damage, which mainly occurs through T-cell mediated mechanisms, but represent a valid biomarker for the diagnosis.[8] Immunosuppressive treatments usually have little effect on the neurological syndrome, that is stabilized or improved by the removal of the tumor.

In recent years, a novel group of neuronal antibodies targeting proteins located on the surface of the cell (S-NAbs) has been described.[1] S-NAbs share similar characteristics that distinguish them from IC-NAbs. First of all, in-vivo accessibility of the antigenic target, located on the surface of the neuron, suggests a potential pathogenic effect of the antibodies, and this has been eventually confirmed for several S-NAb by both in vitro and in vivo experiments.[9,11] Secondly, in most cases, S-NAbs are not associated with the presence of a tumor. Thirdly, most S-NAbs are conformational, and recognize discontinuous epitopes that are strictly dependent on the tertiary structure of the protein. This implicates that specific laboratory assays need to be used in order to properly identify S-NAbs (see page 10).

NMDAR antibodies are the most frequent S-NAbs, and were firstly detected in 2007 in young women with psychiatric symptoms, hypoventilation and ovarian teratoma.[12] After NMDAR antibodies, an increasing number of S-NAbs have been discovered (Table 3), and it is likely that their number will additionally increase over the next years.

A third group of anti neuronal antibodies shares characteristics with both IC-NAbs and S-NAbs. These antibodies, that include for example GAD and amphiphysin antibodies, target intracellular neuronal proteins that are not located in the cytoplasm, but in presynaptic spaces (synaptic intracellular NAbs). Despite their intracellular location, a pathogenic role of the antibodies in altering presynaptic vesicles and transportation has been postulated.[13,14] This could contribute, along with T-cell mediated damage, to the development of the neurological syndrome.

Table 3: Neuronal surface antibodies associated with autoimmune encephalitis (year of discovery and age distribution) (From *Gastaldi et al, 2018*)[15]

Target antigen	Year	Age at presentation	References
NMDAR	2007	Adults>/= Children	Dalmau et al[12]
GLYR	2008	Adults >>Children	Hutchinson et al[16]
AMPAR	2009	Adults	Lai et al[17]
GABA-B R	2010	Adults>>Children	Lancaster et al[18]
LGI1/CASPR2	2010	Adults	Lai et al[19], Irani et al[20]
mGluR5	2011	Adults=Children	Lancaster et al[21]
Dopamine-2 R	2012	Children	Dale et al[22]
DPPX	2013	Adults >>> Children	Boronat et al[23]
GABA-A R	2014	Adults=Children	Petit-Pedrol et al[24]
IgLON5	2014	Adults	Sabater et al[25]
Neurexin-3α	2016	Adults	Gresa-Arribas et al[26]

2.1.2 Autoimmune encephalitis: epidemiology and disease definition

The most common clinical association of S-NAbs is autoimmune encephalitis (AE), an inflammatory disorder of the CNS in differential diagnosis with infectious encephalitis.[27] The epidemiology of AE has changed in recent years, especially with the identification and wide availability of testing for S-NAbs. In a retrospective analysis of large series of patients with suspected encephalitis, Granerod and colleagues found an immune-mediated etiology in 21% of these patients.[27] Moreover, Gable and colleagues, in a series of individuals aged ≤30 years, demonstrated that the frequency of NMDAR encephalitis surpasses that of each viral etiology.[28] A recent study established that the prevalence of AE is 13.7/100000, similar to infectious encephalitis. In addition, the authors found that the incidence of AE increased since 2007, likely because of the increased detection of S-NAbs.[29]

AE is characterized by a wide constellation of symptoms reflecting the synaptic dysfunction the CNS, such as subacute presentation of psychiatric manifestations (delusions, hallucinations, behavioral abnormalities), cognitive impairment and seizures. Different S-NAbs can be associated with different clinical profiles.[30]

Limbic encephalitis (LE) is one of the first forms of AE described, and can be associated to both IC-NAbs and S-NAbs.[31,32] Patients present with a classic triad of behavioral abnormalities, seizures and memory impairment. Brain imaging usually reveals alterations located in the limbic system, and particularly in the temporo-mesial cortex. Many patients have persistent hyponatremia, likely reflecting inappropriate anti-diuretic hormone secretion syndrome (SIADH), and this could provide a useful hint to suspect LE. Many different S-NAbs can be associated with LE including LGI1, CASPR2, $GABA_BR$, AMPAR, DPPX and mGluR5.[32,33]

A peculiar form of AE is NMDAR encephalitis, a disease that was firstly described in young women in association with ovarian teratoma.[12] Additional studies proved that the disorder is in most cases not paraneoplastic, and can occur at any age.[34] In early stages of the disease, patients show psychiatric symptoms (personality disorder and hallucinations) that could be confused with a schizophrenia onset.[35] Within weeks, other neurological manifestations occur including seizures, reduced level of consciousness, cognitive impairment and a wide range of movement disorders.[9] Patients often experience severe dysautonomia and hypoventilation, which could require intensive care support.

In some instances AE can manifest prevalently with epilepsy. For example LGI1 antibodies are associated with typical facio-brachial dystonic seizures (FBDS), brief episodes of unilateral mouth and/or leg contraction of epileptic origin that respond to immunotherapy.[36] $GABA_AR$ antibodies, on the other hand, can be associated with status epilepticus in both adults and children.[37]

When the antigenic target is expressed on both peripheral nerve and CNS, S-NAbs can lead to a combination of AE and PNS manifestations. CASPR2 antibodies target a protein densely distributed both in the limbic system and in the juxtaparanodal area in the nodes of Ranvier. Patients with this antibody can show neuromyotonia, a peripheral nerve hyperexcitability syndrome and/or LE.[20] A combination of LE, sleep disorder, dysautonomia and neuromyotonia is defined as Morvan's syndrome.[38] (see section 4)

2.2 Laboratory detection of neuronal surface antibodies: diagnostic strategy optimization and novel antigen discovery

2.2.1 Rationale and aims of the study

There is poor current agreement on the best laboratory strategy for S-NAbs identification, and only a few studies addressed this issue.[39] Most laboratories rely for routine diagnostic on a commercial kit based on a FCBA (Euroimmun, Lübeck, CCBA) comprehensive of NMDAR, CASPR2, LGI1, $GABA_BR$ and AMPAR 1 and 2. Additionally, similar CCBA are available for rare antigens such as DPPX and IgLON5.

Most of the current evidence on laboratory assays comparison is available for NMDAR antibodies, the most common S-NAb. Gresa-Arribas et al.[40] tested 250 paired serum and cerebrospinal fluid (CSF) samples from patients with NMDAR encephalitis with both IHC and FCBA. The two assays failed to detect antibodies respectively in 8.4% and 31.2% of sera, while all CSF were positive, thus suggesting a slightly higher sensitivity of IHC. To assess whether antibody detection could be improved, the negative sera were retested on both FCBA and LCBA, finding a lower sensitivity of the latter. It has to be considered, though, that in the study the NMDAR encephalitis diagnosis required a positivity on very same tests (i.e. IHC and FCBA) that were evaluated. It is not surprising then that IHC had 100% sensitivity and specificity.

Wandinger et al. performed a comparison between FCBA, IHC and CCBA on 66 patients with NMDAR encephalitis and 381 controls, finding a 100% sensitivity/specificity for both assays, and concluding for a perfect concordance between the assays. On the contrary, other authors reported a low specificity of CCBA, possibly related to difficulties in staining pattern interpretation. [41,42]

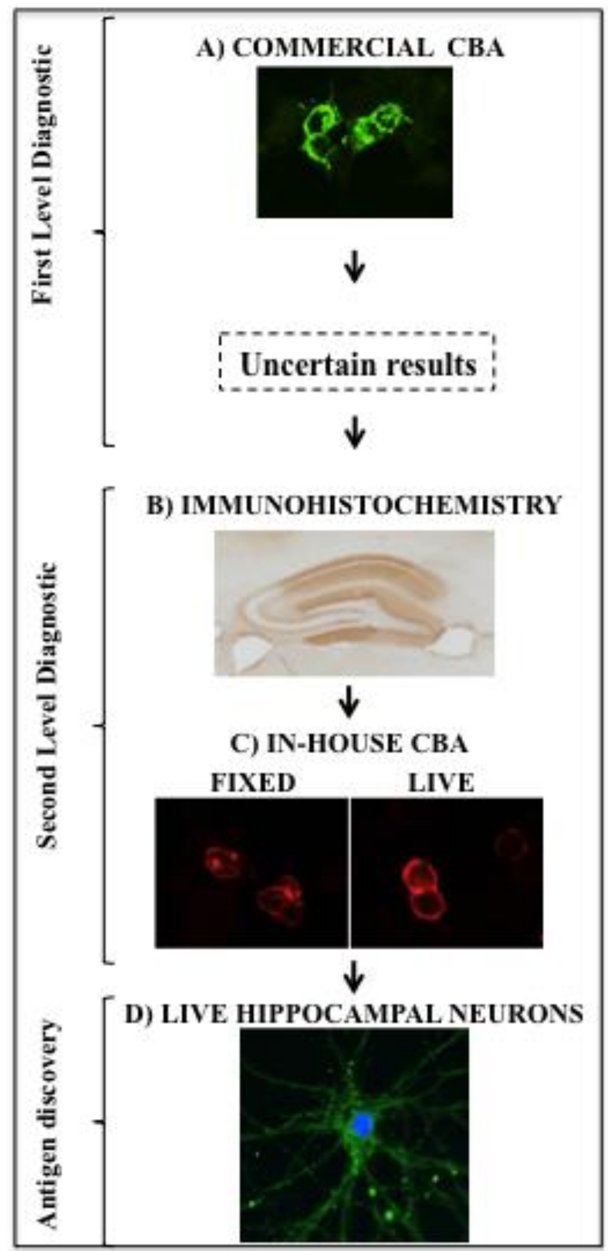
Considering the potential limitation of each test, and the increasing number of S-NAbs identified, concerns regarding the overall performance of CCBA lead to the proposal of a multi-step approach exploiting more than one.[15,39] (Figure 5) McCraken and colleagues[39] analyzed a large cohort of 731 samples sent for NAbs testing, systematically comparing the use of the commercial CCBA with a double step approach combining screening with IHC and, when a neuropilar reactivity was detected, confirmatory test with a wide range of in-house CBA. The second approach

was able to resolve 88/97 undetermined results and to identify additional antibodies in 15 samples, suggesting a better performance of the in-house tests.

Our first aim was to systematically use a combination of commercial and in-house techniques for S-NAbs detection, in order to identify the best laboratory strategy and to quantify its impact of routine diagnostics.

Our second aim was, using IHC as a screening method, to identify uncharacterized reactivities in patients with AE and to identify novel S-NAbs.

Figure 5: laboratory strategy for S-Nab detection (from *Gastaldi et al, 2018*)[15]



A: commercial CBA (Euroimmun, Lübeck) is used by many laboratories that perform a first level diagnostic. Patient's antibodies (green) bind to transfected HEK293 cells expressing the target protein on their surface. If the test provides conflicting results samples should be sent to a second level laboratory able to perform more advanced test. B: Immunohistochemistry on whole rat brain. Patient's antibodies (brown) provide a 'neuropilar' staining localized where the target antigen is more densely expressed (in this example NMDAR antibodies hippocampal staining is shown). C: In house CBAs can be used as a confirmatory test in samples providing a reactivity on murine tissue. As for the commercial CBA, patient's antibodies (red) bind to transfected HEK293 cells, either fixed (left panel) or live (right panel), expressing the target protein on their surface. D: Live hippocampal neurons can also be used as a further test. The binding of autoantibodies to the neurons (green) demonstrates that the target epitope is located on the cell surface. This technique is mainly used in research setting, to identify novel antigenic target of autoantibodies binding to murine tissue but not transfected HEK cells. CBA: cell based assay, HEK293: human embrionic kidney cells 293; NMDAR: N-methyl-D-aspartate receptor.

2.2.2 Methods

2.2.2.1 Study overview

Samples sent to the Pavia neuroimmunology laboratory from January 2017 to June 2018 for S-NAbs testing were prospectively screened using two techniques: a) a CCBA (Euroimmun, Lübeck) mosaic kit including CBAs for NMDAR, LGI1, CASPR2, AMPAR1, AMPAR2 and $GABA_BR$; b) an in-house IHC on rat brain slices optimized for surface antigens (from now on referred to as IHC). In addition to CCBA, when a staining pattern suggestive of synaptic intracellular NAbs was identified, a commercial line blot (Euroimmun, Lübeck) was used for characterization. When a staining suggestive of neuropilar reactivity that could not be characterized by the CCBA was found, the sample was tested with in-house LCBA or FCBA for specific antigens depending on the staining pattern. When all available in-house CBAs failed to characterize the reactivity, and clinical information were available, samples were tested on live hippocampal neuronal cultures. Positive samples, if the clinical information was compatible with a diagnosis of AE, were selected for precipitation experiments and antigen characterization through mass spectrometry.

2.2.2.2 Commercial Cell based assay

CCBA mosaic was performed according to manufacturers' instructions. In brief, samples were diluted in blocking buffer (phosphate buffer saline-PBS+0.02% tween; serum: 1:10) and incubated with a chip containing the cells for 30 minutes. Chips were then rinsed with PBS-0.02% tween and incubated with the appropriate green fluorescent secondary antibody (ready to use, provided by the manufacturer) for 30 minutes at room temperature protected from light. After the final rinsing, cells were mounted with fluorescent mounting medium (provided by the manufacturer).

2.2.2.3 In house Cell based assay (live and fixed cells)

HEK293T cells (IST cell factory, Genoa) were cultured in T75 flasks using Dulbecco's eagle modified essential medium (DMEM) with 10% fetal bovine serum and antibiotics/antimycotics, and then seeded on round glass coverslips (12 mm) in a 6-well plate and grown until 75-80% confluent. Cells were transfected with the

appropriate cDNA (3 ug/well) with lipofectamine 2000 (6 uL/well, Invitrogen) for 24 hours, then washed with fresh DMEM and left in the incubator for 12-24 hours to allow an efficient expression of the target protein. For LCBA, coverslips were transferred to 24 well plates containing patient serum (1:20) or CSF (1:5) diluted with DMEM+1% bovine serum albumin (BSA)+ 0.1 M 01.M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and incubated for 45 minutes at room temperature. Cells were then washed 3 times with DMEM+0.1M HEPES and incubated for 1 hour at room temperature and protected from light with an anti-IgG fluorescent antibody (Jackson scientific anti-Fc or Alexafluor anti Heavy and light chain, both diluted at 1:750). Finally, cells were washed 3 times with DMEM-0.1M HEPES, fixed for 10 minutes in 4% paraformaldehyde (PFA), washed 3 times with phosphate buffer saline (PBS) and mounted on slides using a fluorescent mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI, Dako).

For FCBA, the cells were fixed using 4% PFA for 10 minutes before the staining procedures, then permeabilized with PBS with 0.2% triton for 15 minutes and blocked with PBS-5% bovine serum albumin (BSA) for 1 hour. The remaining steps of the procedure did not differ from the live CBA, apart from the use of PBS-5%BSA as an incubation buffer for both primary and secondary antibody and for the lack of a fixation step before mounting. For both in-house CBA and CCBA results were evaluated under a fluorescent microscope.

2.2.2.4 Immunohistochemistry on rat brain

Unperfused Wistar rats were sacrificed with a lethal injection of tiletamine, and then decapitated. Brains were removed from the skull, cleaned from the meninges, briefly washed in cold PBS and then chopped in half. Afterwards, they were briefly immersed in cold 4% PFA for 1 hour and then transferred to 40% sucrose for 48 hours. The brains were embedded in optimized temperature compound and snap frozen isopentane chilled with liquid nitrogen for 20 seconds. 7 microns–thick slices were cut with a cryostat, collected with polylisine slides and immediately placed at -30°C. Slides were then preserved at -80°C for later use for a maximum time of 3 months. When needed, slides were defrosted, blocked with 0,3% hydrogen peroxide in PBS, washed 3 times and then blocked in 5% normal goat serum (NGS) for 1 hour. Afterwards, slides were incubated with patient’s serum or CSF (1:200 and 1:2 dilutions respectively) overnight at 4°C, then washed and incubated with biotinylated

anti-human IgG (Vector labs) diluted 1:2000 for 2 hours at room temperature. Slides were then washed 3 times with PBS, incubated with avidin-biotin complex (Vector) for 1 hour and washed again 3 times with PBS. Chromogenic reaction was developed using a Diaminobenzidine (DAB) system (SigmaFast, Sigma-Aldrich). Slides were then dehydrated using increasing concentrations of ethanol and Xylene (each step required at least 20 dips; the following steps were used: Ethanol 70%, 100% 1, 100% 2, 100% 3, Xylene 1, Xylene 2, Xylene 3). Finally, slides were mounted using DPX mounting medium (Sigma-Aldrich). Results were assessed using a light microscope. When using serum samples intracellular staining was a frequent finding and was considered as irrelevant. A positive staining was defined as the presence of 'neuropilar staining' (i.e. involving the gray matter matrix where synapses are more densely located)

2.2.2.5 Live hippocampal neuronal cultures (mainly performed at the Barcelona Neuroimmunology laboratory)

A pregnant Wistar rat was sacrificed with a lethal injection of tiletamine and E16-E18, and embryos were retrieved after removing the uterus and placed in a sterile container on ice. Embryos were decapitated, and brains were extracted from the skull and placed in cold hibernate medium supplemented with B27. Each brain was then immersed in a drop of cold hybernate+B27, and hippocampi were dissected and placed in a sterile tube containing hybernate+B27. When all hippocampi were removed, they were transferred to a falcon tube containing a dilution of Hank's balanced salt solution (HBSS) with 1:10 trypsin, and incubated for 15 minutes in water bath at 37°C. 10 ml of HBSS solution were then added to the falcon that was left for incubation in water bath at 37°C for 5 more minutes. Hippocampi were transferred to DMEM at 37°C, and then mechanically separated using a thin glass pipette. Separated neurons were then resuspended in DMEM, counted and seeded in neurobasal +10% fetal bovine serum +B27 in 100 mm petri dish containing 12 mm coverslips at a density of 1000000/1500000000 per petri dish. Plates were gently shaken in order to allow the proper distribution of neurons. Neurons were left in the incubator at 37°C for at least 14-21 days. Every 7 days, 800 uL of neurobasal +10% fetal bovine serum+B27 was added to each petri dish. When neurons were mature, coverslips were transferred to 24 well plates containing serum (dilution 1.100) or CSF (dilution 1:15) diluted with conditioning medium (the medium directly collected from

the petri the neurons grew in) and incubated for 1 hour at room temperature. Neurons were then washed carefully one time with PBS at room temperature, and then fixed for 5 minutes with cold 4% PFA. Neurons were then washed 3 times with PBS, permeabilized for 5 min at room temperature with PBS-0.3% triton and incubated for 1 hour with the secondary antibody (Alexafluor 488 goat anti-human heavy and light, Invitrogen) diluted 1:1000 in PBS+ normal goat serum 5% protected from dark. Slides were then mounted using Prolong Gold fluorescent mounting medium (Vector labs). Results were evaluated using a fluorescence microscope. A staining involving the cell membrane of neurons, often with punctate pattern, was considered as positive.

2.2.2.6 Immunoprecipitation and mass spectrometry (performed at the Barcelona Neuroimmunology laboratory, in collaboration with the University of Pennsylvania)

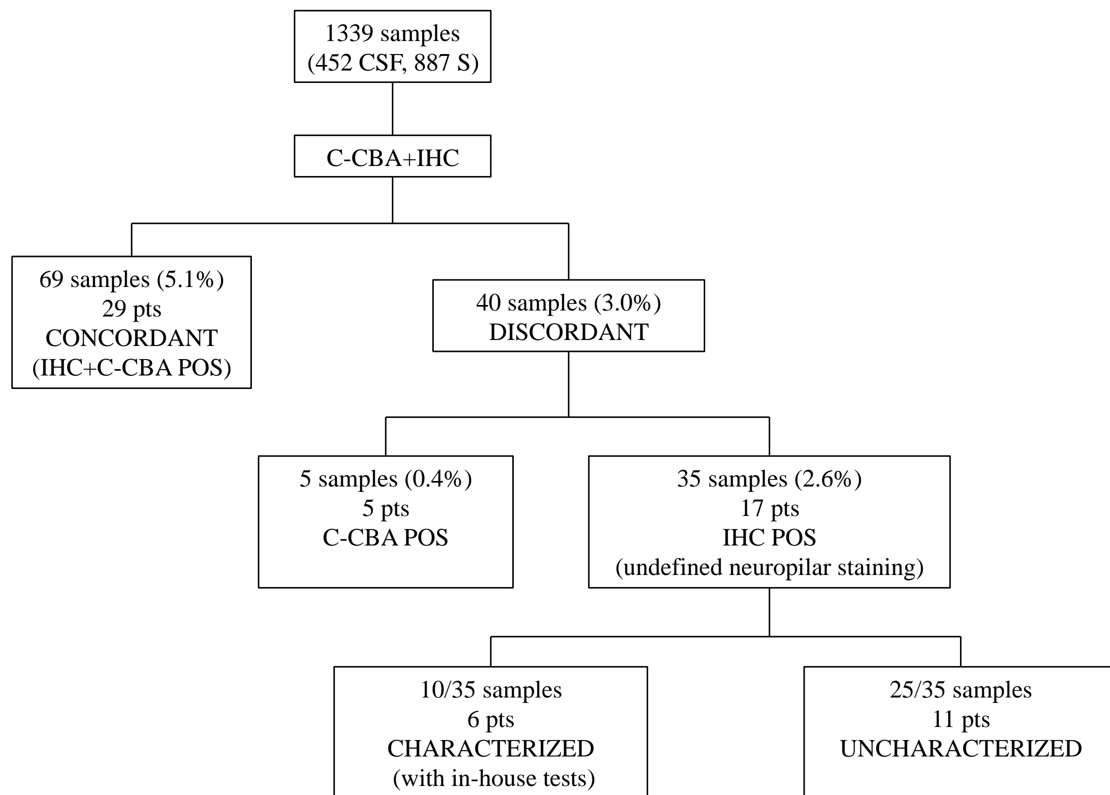
All the steps for the precipitation experiments were performed at 4°C unless specified otherwise. Mature neurons were incubated with patient serum diluted in conditioning medium 1:200. For each precipitation experiment at least 7 p100 petri dishes with neurons were used. Neurons were incubated for 1 hour with light agitation. Patient serum was then aspirated and neurons were incubated with lysis buffer (containing NaCl 150 mM, EDTA 0.5 M, Tris-HCl 100 mM, 10% Deoxycholic Acid and 1% triton) and Noctyl diluted 1:25 for 1 hour on a shaker. Lysate was then collected, mechanically separated and centrifuged to remove cell debris. Supernatant was incubated with A/G protein beads (15 uL for each P100 petri dish) for 2 hours on a shaking plate. Beads were then washed 5 times using lysis buffer, incubated with roti load solution and heated up to 100°C. Beads were then centrifuged and supernatant was separated using an 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in parallel with a positive and negative sample. After the run, the gel was stained with Comassie blue. Specific bands were identified by visual inspection and cut out for mass spectrometry (if no specific bands were identified, the whole gel was used). Samples were sent to the facility in the genomic institute at the Abramson cancer Centre (University of Pennsylvania, USA). Protein bands were digested with trypsin and analyzed with a nanoLC/nanospray/LTQ mass spectrometer.[18]

2.2.3 Results

2.2.3.1 Concordant samples

Figure 6 summarizes the algorithm of the study. In total, 1339 samples were screened for the presence of NAbs (452 CSF and 887 sera) using both IHC and CCBA. Both serum and CSF were always tested on IHC wen available, whilst on CCBA CSF only was used. Sixty-nine/1339 samples (5.1%, corresponding to 29 patients) showed a concordant positivity, meaning that they were positive on IHC and the use of CCBA allowed the characterization of the specific antigens. The most common NAb identified was NMDAR (10 patients), followed by LGI1 (7 patients) and CASPR2 (4 patients). All the patients in this group with NMDAR, LGI1 and CASPR2 antibodies showed a clinical picture compatible with AE. In addition, two patients were co-reactive for LGI1 and CASPR2, both with thymoma and a combination of NMT and LE (Morvan's syndrome), and 1 had $GABA_BR$ antibodies and LE. GAD antibodies and amphiphysin antibodies were identified in respectively 4 and 1 patients, all with stiff person syndrome.

Figure 6: algorithm of the study



CSF: cerebrospinal fluid; S: serum; IHC: immunohistochemistry; CCBA: commercial cell based assay

CCBA positive discordant samples

Forty/1339 samples (3.0%) provided discordant results between IHC and CCBA. Five/34 samples (all sera), corresponding to 5 patients, were positive on CCBA only (NMDAR=3 and CASPR2=2). Among the patients with NMDAR antibodies, one had a muscular disease, and one a progressive multifocal leukoencephalopathy, a brain infection caused by the JC virus. One patient with CASPR2 antibodies had a final diagnosis of multiple sclerosis. No information was available for the remaining 2 patients (one with NMDAR and one with CASPR2 antibodies).

2.2.3.2 IHC positive discordant samples that remained uncharacterized

Twenty-five/35 samples (corresponding to 11 patients) provided a neuropilar staining on IHC that could not be characterized with in-house testing. Clinical information is summarized in table 4. The most common staining patterns were hippocampal (n=3 patients) and diffuse (n=4 patients). Other staining patterns involved the cerebellum (n=1), both cerebellum and hippocampus (n=1), the white matter (n=1) and the striatum (n=1). Five/11 patients were tested on neuronal live cultures resulting negative. Six/11 patients had an alternative diagnosis to AE, whilst 5/11 had possible AE.

Table 4: Clinical features in patient with uncharacterized reactivity on Immunohistochemistry

S: serum; CSF: cerebrospinal fluid; HC: hippocampus; NORSE: new onset refractory status

Pt n	Material	Titre	Pattern	Live hippocampal neurons	Presenting symptoms	Additional features	Final diagnosis
1	S+CSF	S=1:400 CSF=10	HC	Neg	Korsakoff syndrome	Response to steroids	Brain Lymphoma (biopsy proven)
2	S	NA	Diffuse	Not performed	Paresthesia	-	Polyneuropathy
3	S+CSF	S=1:800 CSF:10	Striatum	Neg	Seizures	Dramatic response to PlEx	NORSE
4	CSF	NA	White matter	Not performed	Paraparesis, encephalopathy	Previous HSCT; MOG Abs neg	Demyelinating encephalomyelitis
5	S	S=1:200	Diffuse	Not performed	Weakness in lower limbs	NA	Polyneuropathy
6	S+CSF	NA	Cerebellum	Not performed	Encephalopathy	Multiple brain lesions (possibly ischemic)	Uncertain (possible cardioembolic stroke)
7	S+CSF	S=1:200 CSF= 1:2	Diffuse	Not performed	Encephalopathy	Previous Influenza infection; negative brain MRI	Post-infectious encephalopathy
8	S+CSF	S=1:200 CSF=1:2	HC	Neg	Seizures, confusion	Biopsy showing hippocampal inflammatory infiltrates	Probable AE
9	S+CSF	S=1:200 CSF=1:2	HC+cerebellum	Neg	Slow cognitive decline	Brain MRI: atrophy; normal CSF	Dementia
10	S	NA	HC	Not performed	Psychosis	-	Isolated psychosis
11	S+CSF	NA	Diffuse	Neg	Coma	Brain MRI: brainstem lesions. CSF: >1000 cells	Uncertain (probable CNS infection)

epilepticus; NA: not available; HSCT: homologus stem cell transplantation; MRI: magnetic resonance imaging; NORSE: new onset refractory status epilepticus.

2.2.3.3 IHC positive discordant samples characterized with in-house testing

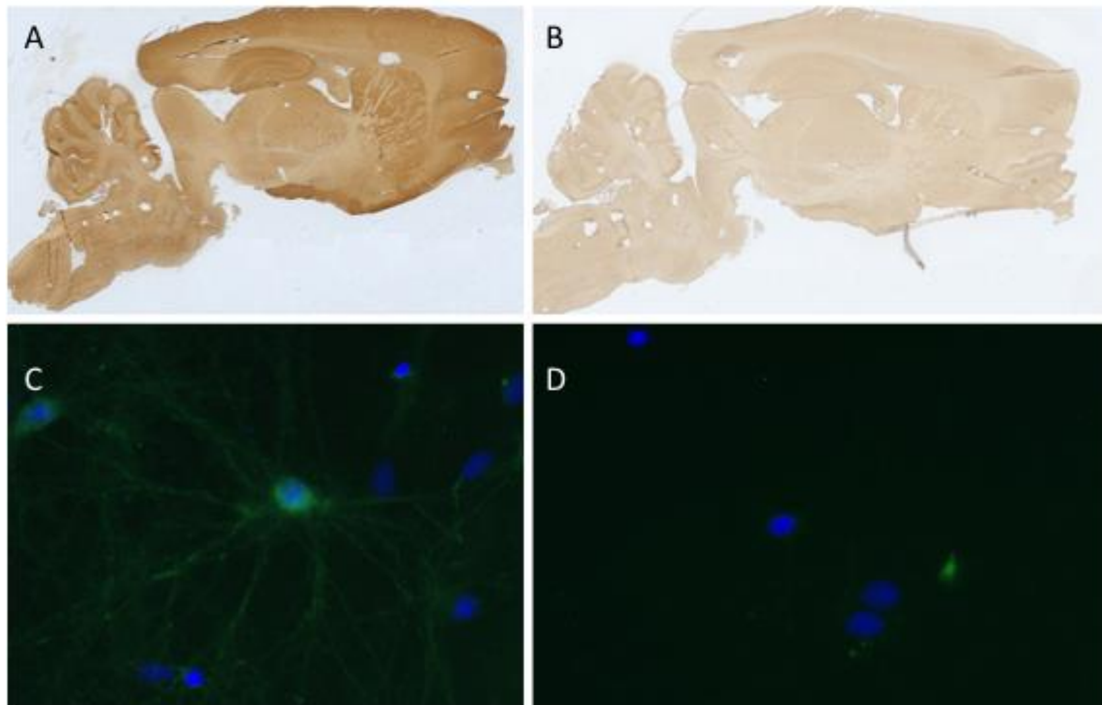
Thirty-five/1339 samples (2.6%) were positive for neuropilar staining on IHC but negative on CCBA. A further diagnostic step using appropriate in-house CBAs and live hippocampal neuronal staining allowed the characterization of S-NAbs in 10/35 samples corresponding to 6 patients (table 5). In 2/6 patients with LE AMPAR antibodies were identified with a LCBA where GluR1 and GluR2 subunit are co-transfected. The same samples resulted negative when tested for AMPAR on CCBA, where GluR1 and GluR2 are transfected separately. One patient had mGluR5 antibodies, usually found in patients with LE. The patient showed an acute onset of aphasia and was diagnosed with an ischemic stroke according to the MRI hippocampal lesion with DWI restriction. The patient did not receive any immunosuppressive treatment, and did not present any other feature suggestive of an inflammatory disorder. Therefore, it is possible that the autoantibodies represented an epiphenomenon, possibly related to the neuronal damage caused by the cerebrovascular disease. The serum from a single patient providing a neuropilar hippocampal staining was negative for all the in-house CBAs compatible with the staining pattern (including NMDAR, $GABA_BR$, $GABA_AR$, mGluR5, AMPAR, LGI1 and CASPR2), but showed strong staining on live hippocampal neuronal cultures. This patient (patient #6, table 5 and figure 7) had a new onset status epilepticus that improved after the administration of intravenous steroids. This serum sample was selected for further precipitation experiments.

Table 5: clinical information of patients characterized with in-house tests

S: serum; CSF: cerebrospinal fluid; LCBA: live cell based assay; FCBA: fixed cell based assay; HC: hippocampus; LE: limbic encephalitis; NA: not available; MRI: agnetic resonance imaging; NMT: neuromyotonia

Pt n	Material	Titre	Pattern	Reactivity	Presenting symptoms	Additional features	Final diagnosis
1	S+CSF	S=1:800 CSF=1:40	HC+cerebellum	LCBA for AMPAR (co-expressed GluR1-2 subunit)	Seizures, memory impairment	Lymphoma	LE
2	S+CSF	NA	Diffuse	LCBA for IgLON5	Chorea, sleep disorders		IgLON5 associated disease
3	S+CSF	NA	HC+Cortex	FCBA for mGluR5	Acute onset aphasia	MRI showing HC ischemic lesion	Uncertain (probable ischemic stroke)
4	S	1:400	HC+Cerebellum	LCBA for AMPAR (co-expressed GluR1-2 subunit)	Fasciculations seizures	Thymoma	NMT and LE
5	S	1:160	HC+Cerebellum	LCBA for LGI1	Fasciculations	Thymoma	NMT
6	S	1:800	HC	Live neurons	Status epilepticus	Precedent traumatic brain injury	Possible AE

Figure 7: immunohistochemistry staining pattern and neuronal staining for patient #6

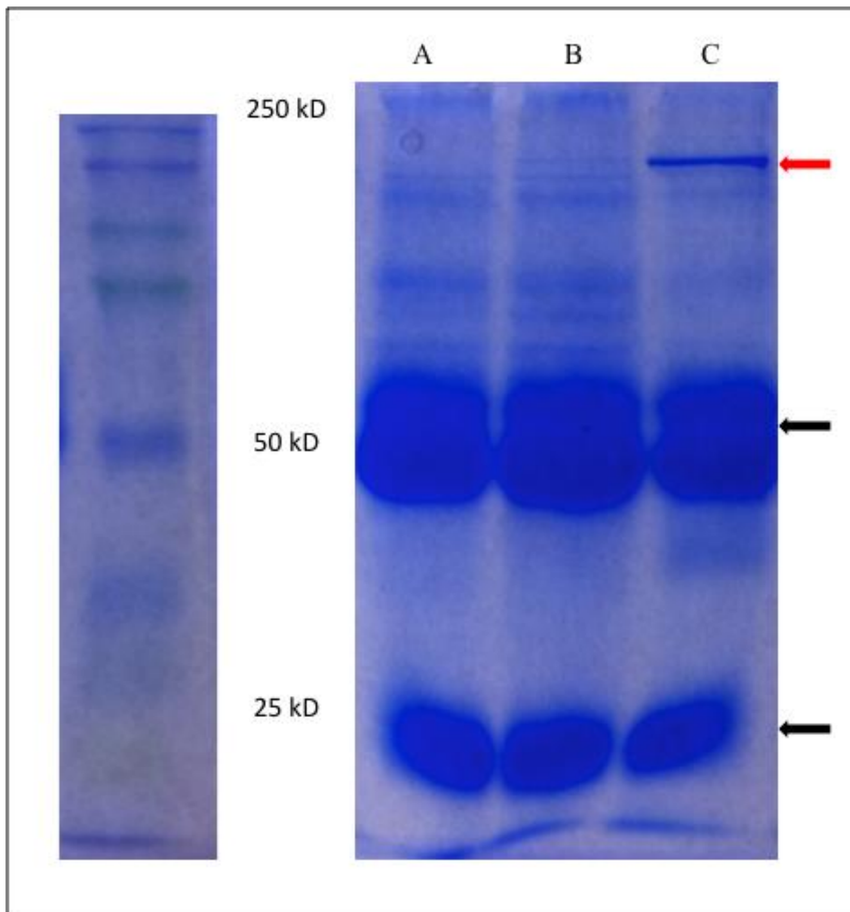


The figure shows the binding of patient #6 serum on IHC (A), where the IgG bind mainly in the neuropilar regions of the hippocampus, on the striatum and on the cortex (brown), and on live neurons (C), where IgG bind on the surface of the neuronal body and dendrites (green). No staining is observed with a negative control on IHC (B) or on live neurons (D). Blue, panel C and D: DAPI.

2.2.3.4 Identification of candidate proteins for novel antigenic targets in AE

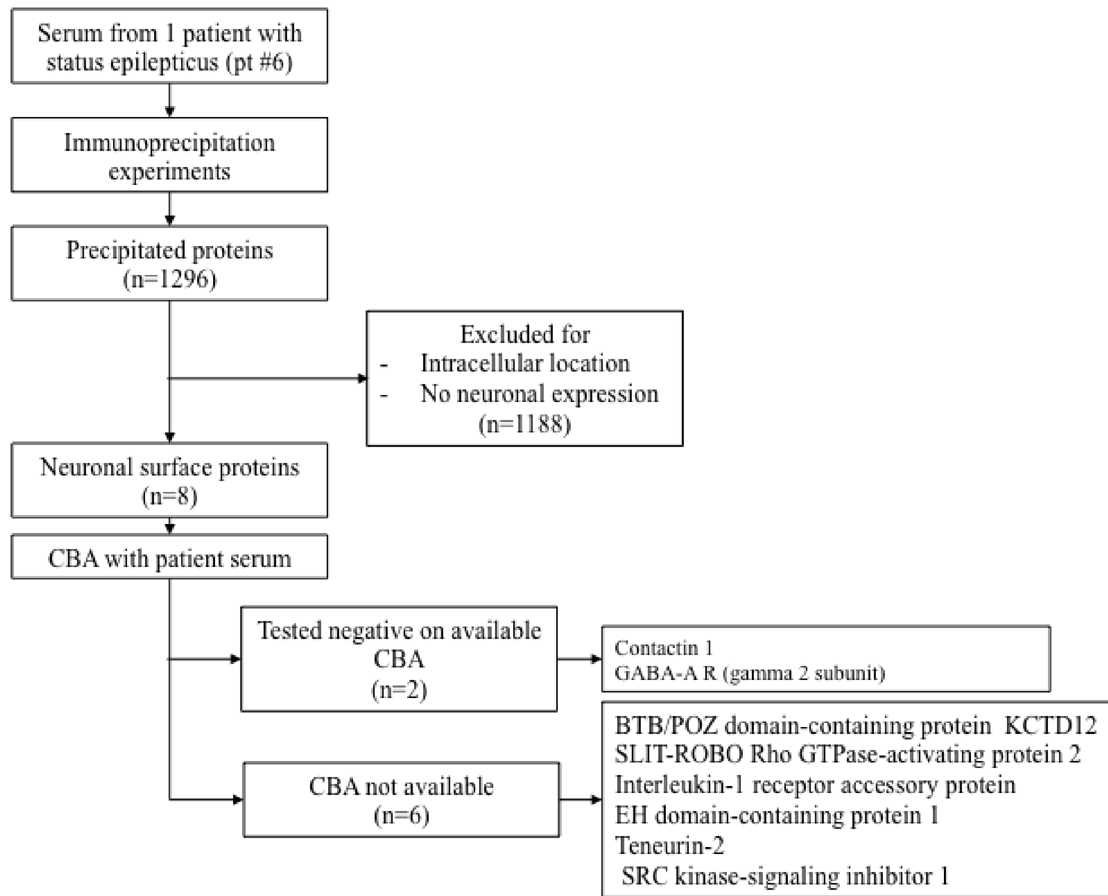
The serum from patient #6 was used for precipitation experiments (see methods section). After separation on SDS-PAGE and blue Comassie staining, the column corresponding to our patient did not show specific additional bands in comparison a positive control (Figure 8). Assuming an insufficient sensitivity of the staining procedure for the identification of low amount of precipitated proteins, we analyzed the whole column with mass spectrometry. The output from this analysis is summarized by the flow-chart in figure 9. At the end of the process, 8 neuronal surface proteins were precipitated. In 2 cases, the precipitated neuronal proteins had been previously identified as antigenic targets in human autoimmune disease, namely contactin-1 (mainly expressed at the paranodal junction of the peripheral nerve) and $GABA_AR$, subunit gamma-2 (a synaptic ionic channel). Contactin-1 antibodies are found in a subgroup of patients with chronic inflammatory demyelinating polyneuropathy, and $GABA_AR$ antibodies are found in a subgroup of AE characterized mainly by seizures and status epilepticus. Patient #6 serum was negative when tested with available CBAs for Contactin-1 and $GABA_AR$. The remaining 6 proteins have never been described as potential autoantibody targets, and their characteristics are summarized in table 6. Only 3/6 are known to be expressed at the synaptic level, and 1/6 is located almost exclusively in the central nervous system. Further experiments, namely to devise CBAs for each protein, will be needed to assess whether the precipitated protein could represent the target of the autoantibodies in our patient.

Figure 8: Precipitation experiment with sample #6



The figure shows the SDS-PAGE separation of the proteins precipitated with patient #6 serum (A), compared with a sample from a healthy control that was used in the same precipitation experiment (B) and a positive control with a serum known to provide a specific band at 230 kD. Since the supernatant contained both the precipitated proteins and the human IgG binding them, the bands provided by the heavy chain at 50 kD and by the light chain at 25 kD are clearly visible with all 3 samples (black arrows). Whilst the positive control provided a clear additional band, as expected (red arrow), the serum from patient #6 did not provide any visible additional band compared with the control. Blue: Comassie blue. Left column: rainbow marker.

Figure 9: precipitated protein from sample #6



CBA: cell based assay.

Table 6: precipitated neuronal surface proteins

Protein name	Gene	Location	Tissue expression	Function
BTB/POZ domain-containing protein KCTD12	KCTD12	Pre and postsynaptic neuronal membrane	Mainly in cochlea and brain	Auxiliary subunit of $GABA_B$ receptors, determines the pharmacology and kinetics of the receptor
Interleukin-1 receptor accessory protein	IL1RAP	Cell membrane and secreted	Liver, skin, placenta, thymus and lung. Isoform 4 is predominantly expressed in brain.	Co-receptor for IL1RL2 in the IL-36 signaling system
SLIT-ROBO Rho GTPase-activating protein 2	SRGAP2	Postsynaptic neuronal membrane, but also dendritic spines, nucleus and cytoplasm	Ubiquitous	RAC1 GTPase activating protein that membranes, regulating cell migration/ differentiation; plays a role in neuronal morphogenesis and migration during development of the cerebral cortex
EH domain-containing protein 1	EHD1	Cell membrane and endosome	Mainly in testis. Low levels in the brain.	ATP- and membrane-binding protein that controls membrane reorganization/tubulation upon ATP hydrolysis.
Teneurin-2	TENM2	Endoplasmic reticulum, plasma membrane, Golgi reticulum	Mainly in heart, followed by brain, liver, kidney and fetal brain	Involved in neural development, regulating the establishment of proper connectivity within the nervous system.
SRC kinase-signaling inhibitor 1	SRCIN1	Post-synaptic neuronal membrane, but also axon and cytoplasm	Mainly brain, but also (among others) in heart, pancreas, gastrointestinal tract	Acts as a negative regulator of SRC, may play a role in neurotransmitter release or synapse maintenance

Main sources: www.uniprot.org and www.proteinatlas.org

2.2.4 Discussion and conclusions

In this study, we implemented an efficient diagnostic strategy of NAbs detection and we prospectively applied it in the routine clinical practice of our laboratory. The use of in-house techniques helped to additionally identify NAbs in 0.7% of the patients tested, and in 12.7% of the positives.

Overall, the performance of the CCBA was good, and was able to identify S-NAb in the majority of cases, showing a good concordance with in-house techniques. In some patients, however, CCBA failed to identify S-NAbs potentially relevant for the patient condition. Partly, this was attributable to the lack of sensitivity of CCBA for some of antigens included in the mosaic kit. AMPAR CCBA seemed particularly critical, and this could be attributed to the different procedure followed by the in-house CBA. In CCBA, the 2 main subunits of AMPAR (GluR1 and GluR2) are expressed separately on the chip. In the LCBA, on the other hand, GluR1 and GluR2 are co-expressed in the same cells, allowing surface interaction. This might suggest relevance for epitopes dependent on the subunit aggregation on the neuronal surface for clinically relevant AMPAR antibodies. It is also possible, though, that the fixation procedures used in the CCBA are sufficient to alter the discontinuous epitopes recognized by conformational AMPAR antibodies and to reduce the sensitivity of the assay.

Even though several groups reported on the risk of false positives with NMDAR CCBA,[41,42] in our hand this was a specific test, but this could be associated to the fact that CSF was mostly used. Indeed, the only false positive reported in our cohort was found using a serum sample. Interestingly, the same sample did not provide any staining on IHC, suggesting that combining CCBA and IHC could be helpful in increasing the diagnostic specificity.

In the whole cohort, we identified only one patient suitable for precipitation experiments. Despite the absence of a definite band on SDS-PAGE, we identified from the mass-spectrometry output 6 potential antigenic target for our patient's autoantibodies. Among the proteins precipitated, some have critical function in synaptic transmission and neuronal morphogenesis. Further experiments will require the implementation of CBA for each protein, followed by the patient's serum testing. Hopefully, this will allow us to finally identify a novel antigenic target for S-NAbs.

In conclusions, we showed that the use of in-house techniques is helpful in clinical practice to identify known NAbs, that could be missed because of a suboptimal performance of the CCBA. In addition, IHC allows also the investigation of uncharacterized relativities and, potentially, the identification of novel antigenic targets for S-NAbs

2.3 Application of the 2016 autoimmune encephalitis diagnostic criteria in the Italian population: a retrospective study

2.3.1 Rationale and aims of the study

In 2016 a panel of experts proposed diagnostic criteria to facilitate the clinic-therapeutic approach to AEs,[43] with two main aims: 1) the definition of a practical algorithm taking into consideration the pivotal role of NAbs; 2) the promotion of timely start of therapies aimed at the removal or reduction of potentially pathogenic circulating autoantibody, considered essential to increase the chances of recovery. The most relevant and possibly critical issue of these diagnostic criteria is linked to the possibility of making a diagnosis of AE (probable or possible AE) if the outcomes of the autoantibodies are not yet available or even if they are negative (NEG-AE).

Since their publication, only a few studies investigated the application of the criteria in clinical practice. Li and colleagues assessed the diagnostic performance on a mixed cohort of previously identified AE and non-inflammatory conditions.[44] They found a high sensitivity of the criteria for possible AE (84%) and high specificity of the criteria for definite limbic encephalitis (LE) (94%) and definite NMDAR encephalitis (98%), but no information was provided on how the S-NAbs were assessed. Kaneko and colleagues examined a cohort of 220 patients with suspect AE screened for the presence of NMDAR antibodies using both IHC and an in house FCBA.[45] They found a sensitivity and specificity of respectively 87.2% and 96.7%. Interestingly, NMDAR antibodies were found in 5 patients not fulfilling the criteria, with incomplete clinical phenotypes such as isolated seizures, post-herpetic encephalitis and demyelinating disorders.

An open issue is represented by the category of antibody-negative AE. Graus and colleagues thoroughly investigated 163 patients with LE for the presence of NAbs, and found 12/163 (7%) that were negative.[46] Four/12 patients had a tumor, and 6/11 improved with immunotherapy.

We studied a large cohort of AE patients, with the main aim of defining the prevalence of each subgroup fulfilling the criteria and of characterizing the subgroup of antibody negative AE.

2.3.2 Methods

2.3.2.1 Study design and inclusion criteria

We retrospectively studied patients referred to 14 Italian neurology/pediatrics departments for suspect autoimmune encephalitis from 2012 to 2016. Patients were included if they fulfilled the inclusion criteria for Possible AE (POSS-AE) adapted from Graus criteria:

- Subacute onset (progression in less than 3 months) of short-term memory defects, altered mental status (impaired level of consciousness, lethargy or change in personality) or psychiatric symptoms
- At least one of the following
  - new-onset CNS focal symptoms
  - epileptic seizures not explained by a previous diagnosis of epilepsy
  - CSF pleocytosis (> 5 leukocytes/mm3)
  - MRI alterations suggestive for AE (hyperintense lesions in T2 involving one or both medial temporal lobes or multifocal lesions involving the gray substance, the white matter or both)
  - Reasonable exclusion of alternative causes. In particular, the following etiologies had to be excluded: infectious encephalitis, prion diseases, toxic/metabolic encephalopathies, cerebrovascular diseases, and brain neoplasm.
- Anti-neuronal antibodies (both IC-NAbs and S-NAbs) tested during the acute phase

Patients with acute disseminated encephalomyelitis (ADEM) or Bickerstaff encephalitis, were not included in the study. Hashimoto encephalopathy, considering the poor clinco-pathological definition of this entity, was included in the study. The presence of onconeural antibodies was not an exclusion criterion.

2.3.2.2 *Screening process and data collection*

Patients inclusion, data collection and revision was performed through a 2 step screening procedure. Firstly, centers participating to the study revised the medical data of patients admitted to the neurology/pediatric wards for suspect encephalitis. For patients fulfilling the inclusion criteria, data regarding clinical presentation, radiological characteristics, laboratory tests, evolution, and final outcome were collected on a database and sent to the coordinating center (Mondino, Pavia). Secondly, a second operator revised the data excluding patients with incomplete information, alternative diagnosis, insufficient data to exclude an alternative diagnosis or inadequate antibody screening.

2.3.2.3 *Neuronal surface antibody testing*

All patients included in the study were tested for the presence of both onco-NAb and S-NAbs. The most widely available testing strategy included indirect immunofluorescence on primate cerebellum followed by a confirmatory line blot for IC-NAbs and a CCBA "mosaic" (including NMDAR, LGI1, Caspr2, AMPAR1-2 and $GABA_BR$) for S-NAbs. Since this was the standard procedure in most centers, it was labelled as "first-line" diagnostic. Some samples were additionally tested with in-house assays including LCBA and FCBA for rare antigens, immunofluorescence/immunohistochemistry on murine tissue optimized for surface antigens and, when a neuropilar staining was detected, with primary neuronal cultures. This set of testing was labelled as "second-line" diagnostic.

2.3.2.4 *Patients classification*

Patients were classified according to the data provided in one of the following diagnostic sub-categories adapted from Graus et al.,[43] based on the presence or absence of S-NAbs: a) seropositive autoimmune encephalitis (POS-AE) including Definite AE (DEF-AE), seropositive definite LE (POS-LE), definite NMDAR encephalitis (POS-NMDAR); b) seronegative AE (NEG-AE) including possible AE (POSS-AE), antibody-negative but probable AE (PROB-AE), definite seronegative LE (NEG-LE), probable NMDAR encephalitis (NEG-NMDAR) and Hashimoto encephalopahty (HE).

Outcome was measured with the modifide Rankin scale (mRS). A poor outcome was defined as an mRS score ≥ 3.

2.3.2.5 *Statistical analysis*

Qualitative variables were summarized as percentages and quantitative variables as median with ranges or interquartile ranges (IQRs). Differences in quantitative variables were tested using t-test or variance analysis (ANOVA) or the analogous nonparametric method (Mann-Whitney test), for qualitative ones through chi-square or Fisher's exact test.

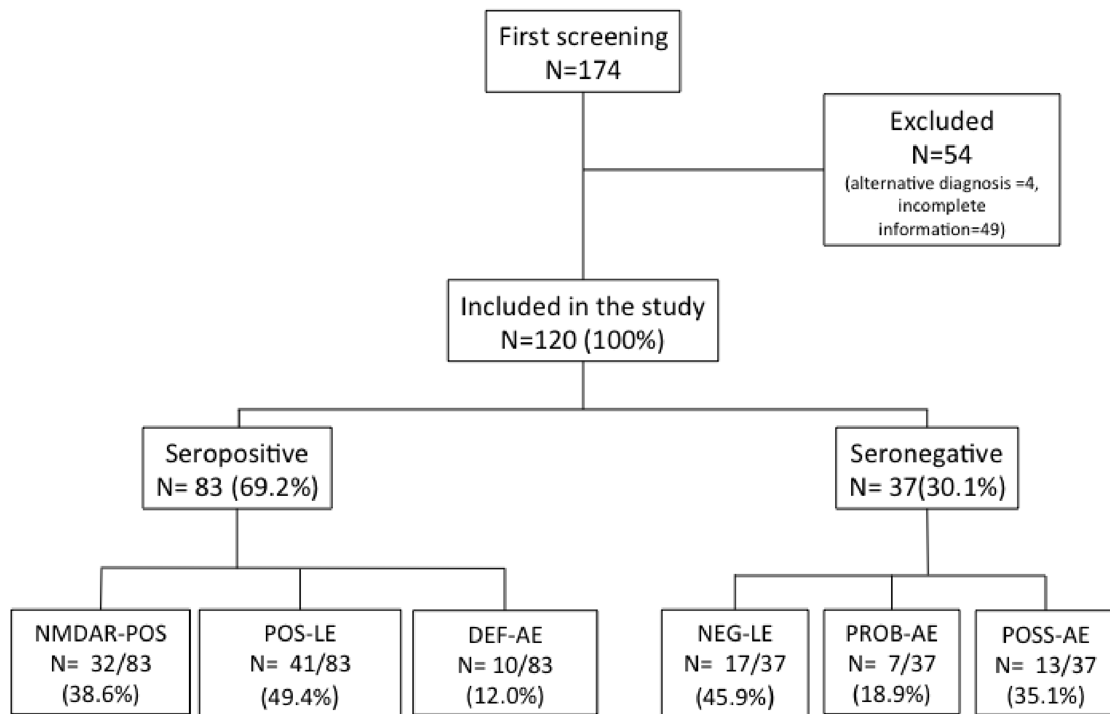
2.3.3 Results

2.3.3.1 *General Data*

A total number of 174 patients fulfilling the criteria for AE according to Graus criteria were included after the first screening performed by the participating centers (Figure 10). Fifty-four of them were excluded after the data revision performed by the coordinating center for incomplete clinical data or incomplete NAbs screening (n=49), and 4 because an alternative diagnosis to AE was identified. At the end of the screening process, 120 patients were finally included in the study, 83 (69.2%) classified as POS-AE and 37 (30.1%) as NEG-AE. The most common clinical syndrome was LE (58 patients, 48.3%), followed by NMDAR-POS (32 patients, 26.7%) (Table 7). Overall, M:F ratio was 1:1, and AE occurrence covered all age range (0-90 years). Age at AE onset followed a bimodal distribution with peaks that reflected the median age of the two most represented clinical groups, namely NMDAR-POS (median 15, range 0-67) and LE (median 62, range 14-90) (Figure 11, panel A). Pediatric patient were mostly distributed in the NMDAR-POS group and in the POSS-AE and PROB-AE groups, whilst were nearly absent in the LE groups (Figure 11, panel B, $p<0.001$). In 19.2 % of the patients, and most frequently in the NMDAR group, the initial diagnostic suspicion was infectious encephalitis ($p<0.001$). A non-inflammatory condition, including a psychiatric or a neurodegenerative disorder, was initially considered in 25/103 patients (20.8%). A tumor was found at the onset of neurological syndrome or during follow-up in 24/120 patients (20.0%). This finding was distributed in all disease groups, and no differences were detected between POS-AE and NEG-AE ($p=0.47$). Three patients had an ovarian teratoma, all with NMDARE. Other common tumors were thymoma (n=6) and neuro-endocrine

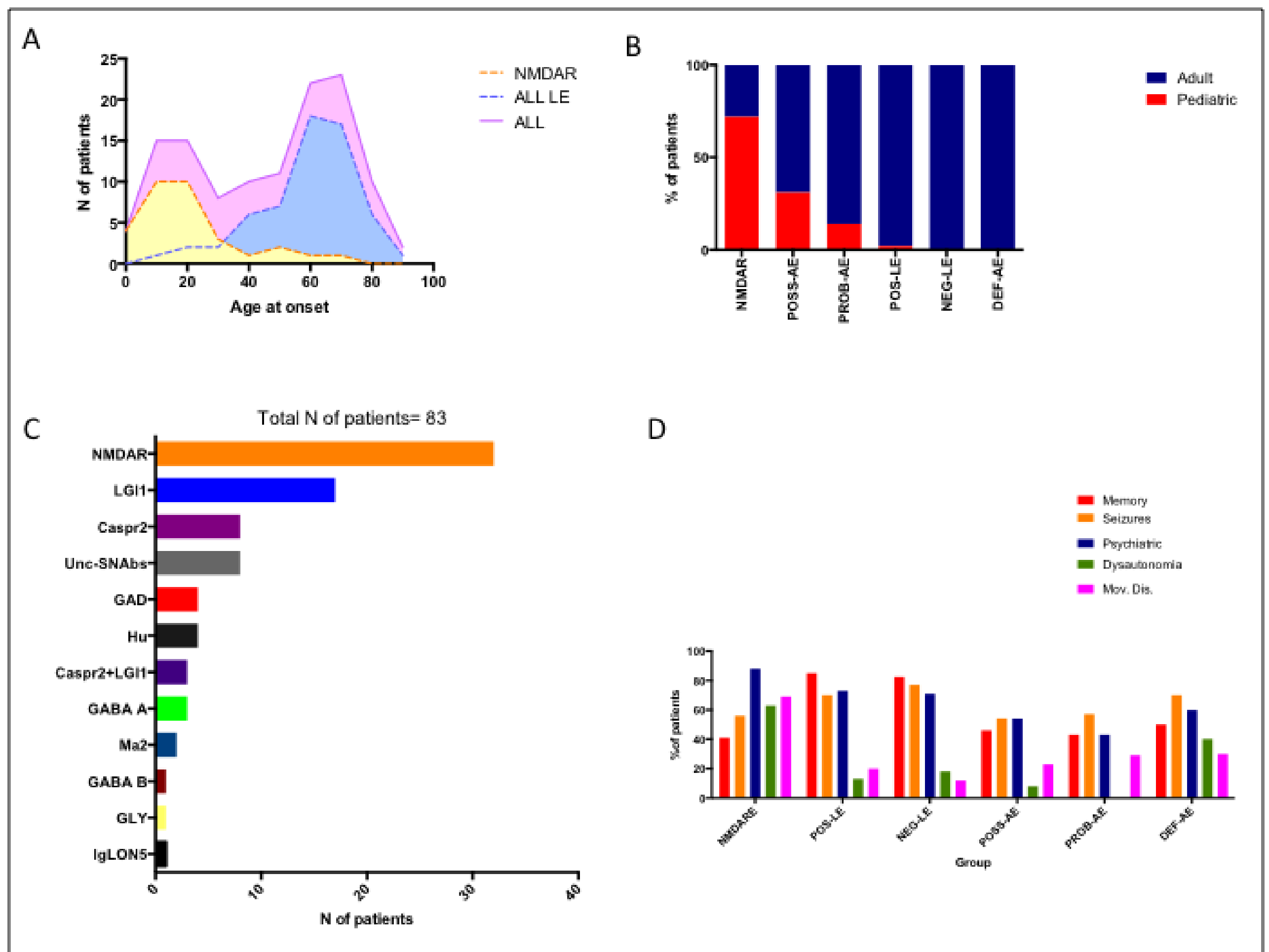
tumor (n=3). Symptoms at presentation were different according to the diagnostic group (Table 7 and figure 11, panel D). Seizures and memory impairment were predominant in the LE group, whilst movement disorders and dysautonomia were more represented in the NMDAR-POS group and in the DEF-AE group ($p<0.001$). Psychiatric symptoms were equally distributed among groups.

Figure 10: algorithm of the study



POS-LE: seropositive limbic encephalitis; DEF-AE: definite autoimmune encephalitis; NEG-LE: seronegative limbic encephalitis; PROB-AE: probable autoimmune encephalitis; POSS-AE: possible autoimmune encephalitis.

Figure 11: clinico-demographic and serological data



A: age distribution in patients with AE followed a bimodal distribution that reflected the median age in POS-NMDAR and LE groups; B: distribution of adults and pediatric patients in the different groups; C: Frequency of S-NAb in the study population; D: distribution of the main clinical features in the different groups. POS-LE: seropositive limbic encephalitis; DEF-AE: definite autoimmune encephalitis; NEG-LE: seronegative limbic encephalitis; PROB-AE: probable autoimmune encephalitis; POSS-AE: possible autoimmune encephalitis.

Table 7: Demographic and clinical characteristics of patients with AE

		POS-AE 83 (69.2)			NEG-AE 47 (38.2)			
	ALL	DEF-AE	NMDAR-POS	POS-LE	NEG-LE	PROB-AE	POSS-AE	p-value
Patients n, (%)	120 (100)	10 (8.3)	32 (26.7)	41 (34.2)	17 (14.2)	7 (5.8)	13 (10.8)	
Male sex, n (%)	58 (48.3)	7 (70.0)	8 (25)	24 (58.5)	7 (41.2)	4 (57.1)	8 (61.5)	0.03
Age (years), median (range)	53 (0-90)	58 (22-80)	15 (0-67)	62 (14-90)	63 (38-82)	37 (8-69)	57 (5-86)	<0.001#
FU duration (months), median (range)	15 (0-192)	14.5 (3-31)	16 (0-63)	21 (0-192)	14 (1-51)	30 (9-57)	6 (0-14)	0.01#
Time to admission (weeks), median (range)	2 (0-788)	1 (0-521)	0.5 (0-63)	1- (0-788)	4 (0-85)	2 (0-118)	6 (0-51)	0.004#
Infectious prodromal event, n (%)	32 (26.7)	3 (30.0)	14 (43.8)	3 (7.3)	4 (23.5)	2 (28.6)	56(46.2)	0.01
Clinical manifestations								
Memory impairment, n (%)	76 (63.3)	5 (50.0)	13 (40.6)	35 (85.4)	14 (82.4)	3 (42.9)	6 (46.2)	<0.001
Psychiatric symptoms, n (%)	86 (71.7)	6 (60.0)	28 (87.5)	30 (73.2)	12 (70.6)	3 (42.9)	7 (53.8)	0.08
Seizures, n (%)	77 (64.7)	7 (70.0)	18 (56.2)	28 (70.0)	13 (76.5)	4 (57.1)	7 (53.8)	0.63
Status epilepticus, n (%)	23 (19.2)	5 (50.0)	6 (18.8)	5 (12.2)	3 (17.6)	3 (42.9)	1 (7.7)	0.05
Movement disorder, n (%)	40 (33.3)	3 (30.0)	22 (68.8)	8 (19.5)	2 (11.8)	2 (28.6)	3 (23.1)	<0.001
Dysautonomia, (%)	33 (27.5)	4 (40.0)	20 (62.5)	5 (12.5)	3 (17.6)	0 (0.0)	1 (7.7)	<0.001
Tumor, n (%)	24 (20.0)	3 (30.0)	4 (12.5)	11 (26.8)	3 (17.6)	2 (28.6)	1 (7.7)	0.47
Initial diagnosis								
Infectious encephalitis(%)	23 (19.2)	1 (10)	6 (18.8)	7 (17.1)	6 (35.3)	1 (14.3)	2 (15.4)	
Epilepsy, n(%)	21 (17.5)	3 (30.0)	5 (15.6)	9 (22.0)	0 (0.0)	3 (42.9)	1 (7.7)	
AE, n(%)	20 (16.7)	2 (20)	2 (6.2)	7 (17.1)	5 (29.4)	1 (14.3)	3 (23.1)	
Other encephalitis, n (%)	15 (12.5)	0 (0.0)	5 (15.6)	3 (7.3)	2 (11.8)	0 (0.0)	5 (38.5)	0.003
Dementia, n (%)	12 (10.0)	0 (0.0)	0 (0.0)	8 (19.5)	3 (17.6)	0 (0.0)	1 (7.7)	
Psychiatric disease, n (%)	13 (10.8)	0 (0.0)	8 (25)	4 (9.8)	0 (0.0)	0 (0.0)	1 (7.7)	
Other, n(%)	16 (13.3)	4 (40.0)	6 (18.8)	3 (7.3)	1 (5.9)	2 (28.6)	0 (0.0)	
mRS ≥ at onset, n (%)	95 (79.8)	10 (100)	31 (96.9)	26 (65.0)	10 (58.8)	6 (85.7)	12 (92.3)	<0.001
Admission to ICU, n (%)	35 (29.2)	5 (50)	17 (53.1)	3 (7.3)	4 (23.5)	3 (42.9)	3 (23.1)	<0.001
Relapses, n(%)	19 (15.8)	1 (10.0)	3 (9.4)	8 (19.5)	2 (11.8)	3 (42.9)	2 (15.4)	0.33
mRS ≥3 at the end of FU, n (%)	37 (31.9)	7 (70.0)	6 (18.8)	10 (25.6)	6 (35.3)	6 (85.7)	2 (18.2)	<0.001
Death, n (%)	6 (5.0)	2 (20.0)	1 (3.1)	3 (7.3)	0 (0.0)	0 (0.0)	0 (0.0)	0.19

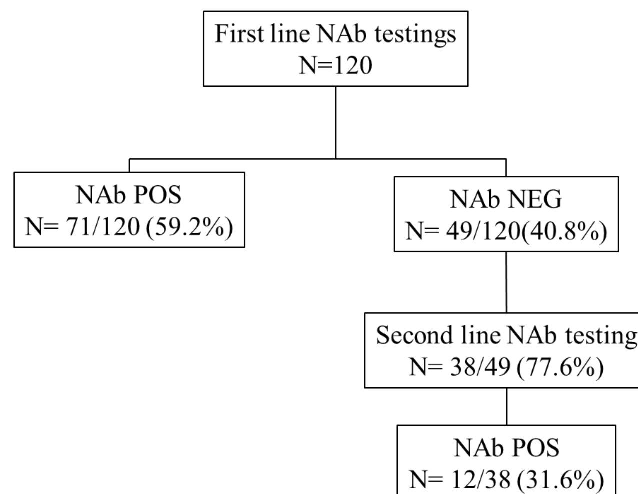
FU: follow-up; AE: autoimmune encephalitis; modified Rankin scale. #Mann-Whitney test.

2.3.3.2 *Anti-neuronal antibodies*

In all patient both serum and CSF were tested for the presence of NAbs, except for 1 patient with LE and LGI1 antibodies where only serum was available. Eighty-three patients had NAbs, and the most frequently identified was NMDAR (n=32), followed by LGI1 (n=20) and CASPR2 (n=12). Six patients had onco-NAb (Hu n=4, Ma2 n=2) and 4 patients had synaptic intracellular NAbs (GAD n=4) (Figure 11, panel C).

NAbs testing procedures are summarized in figure 12. Overall, in 71/120 patients (59.2%) NAbs were identified and characterised using a first line test. Among the remaining negative patients, 38/49 (77.6%) underwent a second line testing, and this was helpful in identifying NAbs in 12/38 patients (31.6% of the patients tested). In one patient with LE the first line test failed to identify AMPAR antibodies, that were detected with a LCBA, whilst in 5 patients the S-NAbs identified were not included in the first line of screening ($GABA_AR$ n=3, IgLON5 n=1 and GLYR n=1). The remaining 6 patients had antibodies directed against an unknown neuronal surface proteins confirmed by binding to live neurons. All these 6 patients fulfilled the criteria only for POSS-AE, but the detection of a S-NAb allowed their classification in the DEF-AE group. Finally, sera from 2 patients showed a neuropilar reactivity on murine tissue, but could not bind to live neurons. Considering this finding of uncertain clinical meaning, the patients were classified as POSS-AE and PROB-AE.

Figure 12: First and second level neuronal antibody testing in patients with autoimmune encephalitis



NAb: neuronal antibodies

2.3.3.3 Patients with DEF-AE

Ten patients had S-NAbs that were not associated with either NMDAR encephalitis or LE. (Table 8) All patients were adults (median age: 58, range 22-80). Three/10 patients with DEF-AE had tumors, one patient with a thymoma and a typical $GABA_A R$ encephalitis, one with a thymic carcinoma, CASPR2 antibodies and an ataxic syndrome and one patient with uncharacterized antibodies and new onset seizures.

Seizures were common, and were found in 7/10 patients. Notably, in 5/10 patients seizures were the main neurological presentation and in 5/10 patients evolved to status epilepticus. One patient with a complex movement disorder resembling a chorea and a sleep disorder was identified as an IgLON5-associated syndrome, and one patient had GLYR antibodies associated with a combination of brainstem encephalitis and severe L-dopa responsive dystonia diagnosed as progressive multifocal encephalomyelitis with rigidity and myoclonus (PERM). MRI revealed unilateral mesial temporal lobe involvement in 3/10 patients, and in one $GABA_A R$ positive patient multifocal T2 hyper-intense lesions involving both the gray and the white matter. All patients in the DEF-AE group had high disability during the acute phase, and half of them required an admission to intensive care unit. A the end of follow-up, 70% of patients had a poor outcome. Relapses were rare, and affected only 1/10 patients.

Table 8: clinical and serological characteristics in patients with DEF-AE

Pt N	Sex	Age at onset	S-NAb	Main clinical syndrome	CSF	Brain MRI	Tumor
1	M	41	GLY	PERM	OCB	nomal	
2	M	53	Caspr2	Ataxia, cognitive dysfunction	normal	normal	Thymic carcinoma
3	M	80	GABA-A	Seizures and status epilepticus	normal	Multifocal T2 hyper-intense lesions (gray+white matter)	Thymoma
4	F	71	IgLON5	Choreiform movement disorder, Sleep disorder	NA	normal	-
5	M	56	Uncharacterized (neurons +)	Seizures and status epilepticus	2 LMN/mm^3	normal	-
6	M	22	Uncharacterized (neurons +)	Seizures and status epilepticus	normal	T2 lesion in medial temporal lobe (unilateral)	-
7	F	24	Uncharacterized (neurons +)	Seizures and Status epilepticus	35 LMN/mm^3	T2 lesion in medial temporal lobe (unilateral)	-
8	M	60	Uncharacterized (neurons +)	Brainstem encephalitis	normal	normal	-
9	M	64	Uncharacterized (neurons +)	Post-HSV encephalitis with seizures and hallucinations	OCB	T2 lesion in medial temporal lobe (unilateral)	-
10	F	65	Uncharacterized (neurons +)	Seizures	normal	normal	NSCLC

M: male; F; female; S-NAb: surface neuronal antibody; CSF: cerebrospinal fluid; MRI: magnetic resonance imaging; PERM. Progressive encephalomyelits with rigidity and myoclonus; LMN: lymphomonocytes; OCB: oligoclonal bands; HSV: herpes simplex virus; NSCLC: non small cell lung carcinoma.

2.3.3.4 Patients with POS-NMDAR

All the 32 patients with NMDAR antibodies had a clinical phenotype compatible with the diagnosis of POS-NMDAR. Clinical, MRI and CSF data of POS-NMDAR patients are summarized in table 9. The most common presenting features were psychiatric symptoms and altered mental status (respectively 87.5% and 78.1% of patients). Severe and potentially life-threatening manifestations such as central hypoventilation (37.5% of patients) and status epilepticus (18.8% of patients) were not uncommon, and half of the patients required admission to the intensive care unit. Twenty-two/32 patients (68.8%) showed movement disorders in the disease course, manifesting in 10/22 patients as diskynesias. Other rarer movement disorders included chorea (n=1), dystonia (n=1) and brief symmetric myoclonic jerks involving the shoulders with a normal EEG (n=1). No information regarding the movement disorder pattern in the remaining patients were available. In order to diagnose NMDAR encephalitis in the absence of antibodies, a complete clinical picture with at least 4/6 core features is required.[43] Interestingly, in our cohort only 56.3% of the patients showed this full clinical picture. The remaining patients showed 2-3/6 (11 patients, 34.4%) or even only 1/6 (3 patients, 9.4%) core features. Age at presentation did not affect the number of core feature shown by the patients.

Table 9: clinical and paraclinical features in patients with POS-NMDAR

POS-NMDAR patients, n (%)	32 (100)
Previous HSV encephalitis, n (%)	1 (3.1)
Clinical manifestations	
Altered mental status, n (%)	25 (78.1)
Psychiatric symptoms, n (%)	28 (87.5)
Focal symptoms, n (%)	10 (31.3)
Seizures, n (%)	18 (56.3)
Status epilepticus, n (%)	6 (18.8)
Movement disorders, n (%)	22 (68.8)
Autonomic dysfunction, n (%)	20 (62.5)
Central hypoventilation, n (%)	12 (37.5)
Hyponatremia, n (%)	6 (18.8)
N of NMDAR encephalitis core features ≥ 4, n (%)	18 (56.3)
Tumor, n (%)	4 (21.5) (3 ovarian teratoma, 1 lymphoma)
ICU, n (%)	17 (53.1)
CSF analysis	
Altered CSF, n (%)	25 (78.1)
Cell count, median (range)	12 (0-360)
OCB, n (%)	15 (46.9)
Brain MRI	
Altered MRI, n (%)	9 (28.1)
Limbic system involvement, n (%)	7 (21.9)
Altered EEG, n (%)	30 (93.8)
Epileptiform alterations, n(%)	9 (28.1)
Extreme Delta brush, n (%)	4 (12.5)

HSV: herpes simplex virus; ICU: intensive care unit; CSF: cerebrospinal fluid; OCB: oligoclonal bands; MRI: magnetic resonance imaging; EEG: electroencephalography.

2.3.3.5 Patients with LE

LE was the most common clinical syndrome, and POS-LE was more frequent compared to NEG-LE (respectively 41 and 17 patients). The most common antibody in the POS-LE group was LGI1 that was found in 20/41 patients (48.8%), 3 with concomitant CASPR2 antibodies. Two/3 patients with concomitant LGI1 and CASPR2 antibodies had a sleep disorder and neuromyotonia and were diagnosed with Morvan's syndrome, and 1 had concomitant neuromyotonia and myasthenia gravis. The presence of faciobrachial dystonic seizures (FBDS) was reported in 5/20 patients with LGI1 antibodies (25%). An inflammatory CSF analysis was found in half of the patients (48.8%), and an altered EEG in up to 89.4%. Temporal slow waves were the most common alteration described (72.3% of patients). As expected on the basis of the diagnostic criteria, the presence of bilateral hippocampal involvement in brain MRI was found more frequently in NEG-LE compared to POS-LE (respectively 42.5% vs 82.4%, p=0.01). Two patients with NEG-LE did not show a bilateral involvement with the brain MRI, but this was established with a PET-scan. Strikingly, POS-LE and NEG-LE did not show any significant differences in clinical presentation, tumor frequency or CSF profile (see table 10). In addition, relapses occurred similarly in both groups (19.5% of patients in POS-LE vs 11.8% of patients in NEG-LE, p=0.48).

The tumor type differed between POS-LE and NEG-LE. In POS-LE, 11/41 patients (26.8%) had a tumor, and the most common were thymoma (n=4) followed by neuroendocrine tumor (NET, n=3). Other rare tumors were testicular cancer (n=1), NSCLC (n=1), Lymphoma (n=1) and basalioma (n=1). In NEG-LE, 3/17 patients had a tumor (17.6%); this included a renal carcinoma (n=1), a mucoepidermoid carcinoma of the jaw (n=1) and breast cancer (n=1).

Table 10: clinical and paraclinical features in patients with antibodynegative and positive limbic encephalitis

LE patients, n (%)	ALL LE 58 (100)	POS-LE 41 (100)	NEG-LE 17 (100)	P=value
Clinical manifestations				
Memory impairment, n (%)	49 (84.5)	35 (85.4)	14 (82.4)	0.77
Psychiatric symptoms, n (%)	42 (72.4)	30 (73.2)	12 (70.6)	0.84
Seizures, n (%)	41 (71.9)	28 (70.0)	13 (76.5)	0.62
Status epilepticus, n (%)	8 (13.8)	5 (12.2)	3 (17.8)	0.68*
Movement disorders, n (%)	10 (17.2)	8 (19.5)	2 (11.8)	0.71*
Autonomic dysfunction, n (%)	8 (13.8)	5 (12.2)	3 (17.6)	0.68*
Hyponatremia, n (%)	13 (22.4)	11 (26.8)	2 (11.8)	0.31*
Tumor, n (%)	14 (24.1)	11 (26.8)	3 (17.6)	0.52*
CSF analysis				
Altered CSF, n (%)	27 (54.0)	19 (57.6)	8 (47.1)	0.48
Cell count, median (range)	2 (0-186)	2 (0-186)	2 (0-50)	0.44#
Cell count >/=5, n (%)	15 (25.9)	9 (22.0)	6 (35.3)	0.29
OCB, n (%)	14 (24.1)	11 (26.8)	3 (17.6)	0.52*
Brain MRI				
Altered MRI, n (%)	43 (74.1)	26 (63.4)	17 (100.0)	0.11
Monolat. limbic lesions, n (%)	8 (14.0)	6 (15.0)	2 (11.8)	0.01
Bilat. limbic lesions	35 (60.3)	20 (48.8)	15 (88.2)	
Altered EEG, n (%)	44/48 (91.7)	31/34 (91.2)	13/14(92.9)	0.98
Temporal slow waves, n (%)	39/48 (81.2)	27/34 (79.4)	12/14(85.7)	0.61
Epileptiform alterations, n (%)	26/48 (54.2)	18/34 (52.9)	8/14 (57.1)	0.79

LE: limbic encephalitis; CSF: cerebrospinal fluid; OCB: oligoclonal bands; MRI: magnetic resonance imaging; EEG: electroencephalography. *Fisher's exact test; #Mann-Whitney test.

2.3.3.6 Patients with probable and possible seronegative AE

A total number of 20/120 patients were classified as either PROB-AE (n=7) or POSS-AE (n=13)(Table 11 and 12). The most common clinical syndrome was an LE-like phenotype (with a combination of memory impairment, behavioral abnormalities and seizures) found in 11/20 patients. Eleven/20 patients (55%) had seizures, and status epilepticus was found in 4/20 patients (20%). An inflammatory CSF was found in 13/20 patients (65%), and OCB were detected in 8/20 patients (40%). Brain MRI showed unilateral involvement of temporomesial structures in 4/20 patients (20%), in one case with additional involvement of the thalamus and of the subcortical white matter. One patient had bilateral temporomesial lesions with additional basal ganglia involvement. An altered brain MRI, along with inflammatory alterations in the CSF, was found in all 7 patients with PROB-AE, as expected according to the classification criteria. Five/20 patients (25%) showed relapses during follow-up, and a tumor was found in 3/20 (15%) and included breast cancer (n=1), pancreatic NET (n=1) and testicular cancer (n=1).

Table 11: clinical and paraclinical characteristics of patients with PROB-AE

Pt N	Sex	Age at onset	Treatment	Main clinical syndrome	CSF	Brain MRI	Response to treatment	Course	mRS at the end of FU	Tumor
1	M	27	IvMP, IvIG, PlEx	LE-like	7 LMN/mm^3, OCB	White matter multifocal lesions and atrophic evolution	No	Monophasic	5	-
2	F	29	IvMP,Pr	Post yellow fever vaccination encephalitis with dyskinesia	53 LMN/mm^3, OBC	Multifocal grey matter lesions (predominantly in cerebellum and occipital cortex)	Yes	Monophasic	0	-
3	M	55	IvMP,Pr, IvIG	LE-like with status epilepticus	18 LMN/mm^3	T2 hyperintense MTL lesion (bilateral); basal ganglia involvment	No	Monophasic	3	-
4	F	69	Pr, PlEx	LE -like	OCB	Periependimal bilateral lesions disappeared after PlEx	Yes	Monophasic	3	Breast cancer
5	M	37	IvMP, IvIG, PlEx, Rtx	Seizures	4 LMN/mm^3, OCB	T2 hyperintense MTL lesion (monolateral)	No	Relapsing	3	Testicular cancer
6	F	37	IvMP, IvIG	Predominant behavioral abnormalities	60 LMN/mm^3	Multifocal white matter abnormalities	No	Relapsing	3	-
7	M	8	IvMP, IvIG, PlEx, CPA	Seizures and status epilepticus	OCB	T2 hyperintense MTL lesion (monolateral)	Yes	Relapsing	3	-

CSF: cerebrospinal fluid; MRI: magnetic resonance imaging; mRS: modified Rankin scale; FU: follow-up; IvMP: intravenous methylprednisolone; IvIG: intravenous immunoglobulins; PlEx: plasma exchange; LE: limbic encephalitis; LMN: lymphomonocitoid; OCB: oligoclonal bands; Pr: oral prednisone; MTL: mesial temporal lobe; Rtx: rituximab; CPA: cyclophosphamide.

Table 12: clinical and paraclinical characteristics of patients with POSS-AE

Pt N	Sex	Age at onset	Treatment	Main clinical syndrome	CSF	Brain MRI	Response to treatment	Course	mRS at the end of FU	Tumor
1	M	5	IvMP, Pr, IvIg, PlEx	Seizure and status epilepticus	Normal	Normal at onset; during follow-up bilateral lesions in pulvinar, thalami and claustrum	Yes	Monophasic	0	-
2	F	15	IvMP, Pr	LE-like	Normal	Normal	Yes	Monophasic	2	-
3	F	57	IvMP	LE-like with seizures and status epilepticus	70 LMN/mm3	Normal	Yes	Monophasic	2	-
4	F	69	IvMP, IvIG	Le-Like	Normal	T2 hyper-intense MTL lesion (monolateral)	Yes	Monophasic	1	Pancreatic NET
5	M	74	IvIg	LE-like, sleep disorder (insomnia)	Normal	Normal	Yes	Monophasic	2	-
6	M	75	PlEx	LE-like	16 LMN/mm3	Normal	Yes	Monophasic		-
7	F	76	Pr, IvIg	Le-Like+ limbs myoclonic jerks	Normal	normal	Yes	Monophasic	2	-
8	M	86	IvMP, PlEx	LE-Like	4 LMN/mm3	T2 hyper-intense MTL lesion -(monolateral)	Yes	Monophasic	1	-
9	M	6	IvMP, IvIg	Encephalitis with seizures and status epilepticus	24 LMN/mm3	Normal	Yes	Relapsing	2	-
10	M	30	IvMP, Pr, IvIg,	Encephalopathy with myoclonic jerks in lower limbs and tremor	19 LMN/mm3 OCB	Multifocal grey and white matter lesions	Yes	Relapsing	1	-
11	F	11	IvMP, Pr, IvIg, CPA	Behavioral alterations and movement disorder (dystonia)	18 LMN/mm3	Normal at onset; During FU T2 lesion in cerebellar dentate nucleus	Yes	Monophasic	3	-
12	M	49	No treatment	Post influenza-virus encephalopathy with seizures	10 LMN/mm3 OCB	Normal	NA	Monophasic	3	-
13	M	75	No treatment	LE-Like	Normal	T2 hyper-intense mesial temporal lobe lesion (monolateral) + involvement of thalamus	Yes	Monophasic	NA	-

CSF: cerebrospinal fluid; MRI: magnetic resonance imaging; mRS: modified Rankin scale; FU: follow-up; IvMP: intravenous methylprednisolone; IvIG: intravenous immunoglobulins; PlEx: plasma exchange; LE: limbic encephalitis; LMN: lymphmonocytes; OCB: oligoclonal bands; Pr: oral prednisone; Rtx: rituximab; CPA: cyclophosphamide; NET: neuroendocrine tumor

2.3.3.7 Treatment, relapses and disability

Most patients received an immunosuppressive or immunomodulatory treatment. A first line of treatment was given to 111/120 patients, most commonly with steroids (84.2% of patients). Other common treatments were intravenous immunoglobulin (IvIg) (57% of patients) and plasma exchange (PlEx) (37.5% of patients). A combination strategy with 2 or more first line treatment was given to 84/111 treated patients (75.7%). A second line of treatment for the first event was given to 28/120 patients (23.3%), with either rituximab (n=13), cyclophosphamide (n=12) or both (n=7). Additional drugs for long-term immunosuppression included mycophenolate mophetyl in 6 patients and azathioprine in 6 patients. Nine patients did not receive any treatment, 5/9 in the NEG-AE group. The therapeutic strategy differed between POS-AE and NEG-AE. Only 4/37 NEG-AE patients (10.8%) received either rituximab or cyclophosphamide, compared to 24/59 with POS-AE (28.9%, p=0.03, figure 13, panel A and figure 14).

A satisfactory response to the treatment was reported in 94/111 treated patients, more frequently in the POS-AE compared to the NEG-AE group (respectively 88% of patients vs 56.8%, p=0.001, figure 13, panel D). In particular, only 57.1% of patients with NEG-LE and 42.9% of patients with PROB-LE had a satisfactory response. Treatment efficacy was dependent on the timing of administration, and in responders time to treatment was shorter (median: 4 weeks, IQR: 2-23) compared to non-responders (median 46 weeks, IQR:3-59, p=0.04, Figure 13, panel E). Patients with POS-AE were more likely to be treated within 6 months since the onset of the neurological disease (p=0.01, figure 13 panel F).

In the acute phase of the disease, 79.8% of the patients had an mRS score ≥ 3, and 35% required an admission to the intensive care unit (ICU). Severely ill patients were more frequent in the POS-NMDAR group (31/32, 96.9%), in the DEF-AE group (10/10, 100%) and in the PROB-AE/POSS-AE group (18/20, 90%)(p=0.001).

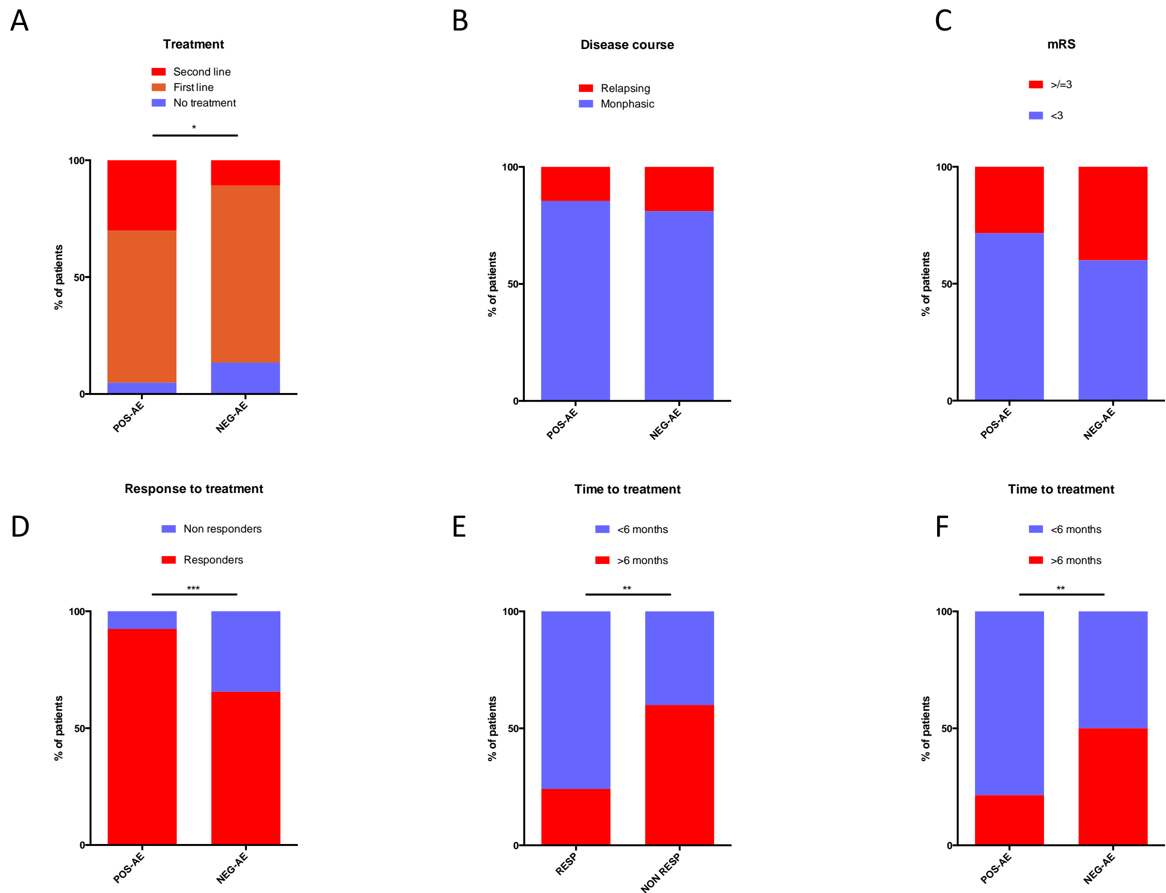
At the end of follow-up, 31.9% of patients had a poor outcome defined as a mRS score ≥ 3. Patients with poor outcome were prevalent in the PROB-AE group (85.7% of patients) and in the DEF-AE group (70% of patients) compared to the other groups (p=0.001). Patients with NEG-AE group had a slightly worse outcome, but this did not reach statistical significance (p=0.22) (figure 13, panel C). Factors associated with a bad outcome were the presence of a status epilepticus (p=0.02) and the type of

treatment received: untreated patients had a significantly worse follow-up, and 6/8 (75%) had a mRS score ≥ 3 (p=0.02).

Six patients died during follow-up, 5 with POS-LE and 1 with POS-NMDAR. Four/6 patient died because of the progression of an underlying tumor, and 2 for complications of the neurological disease.

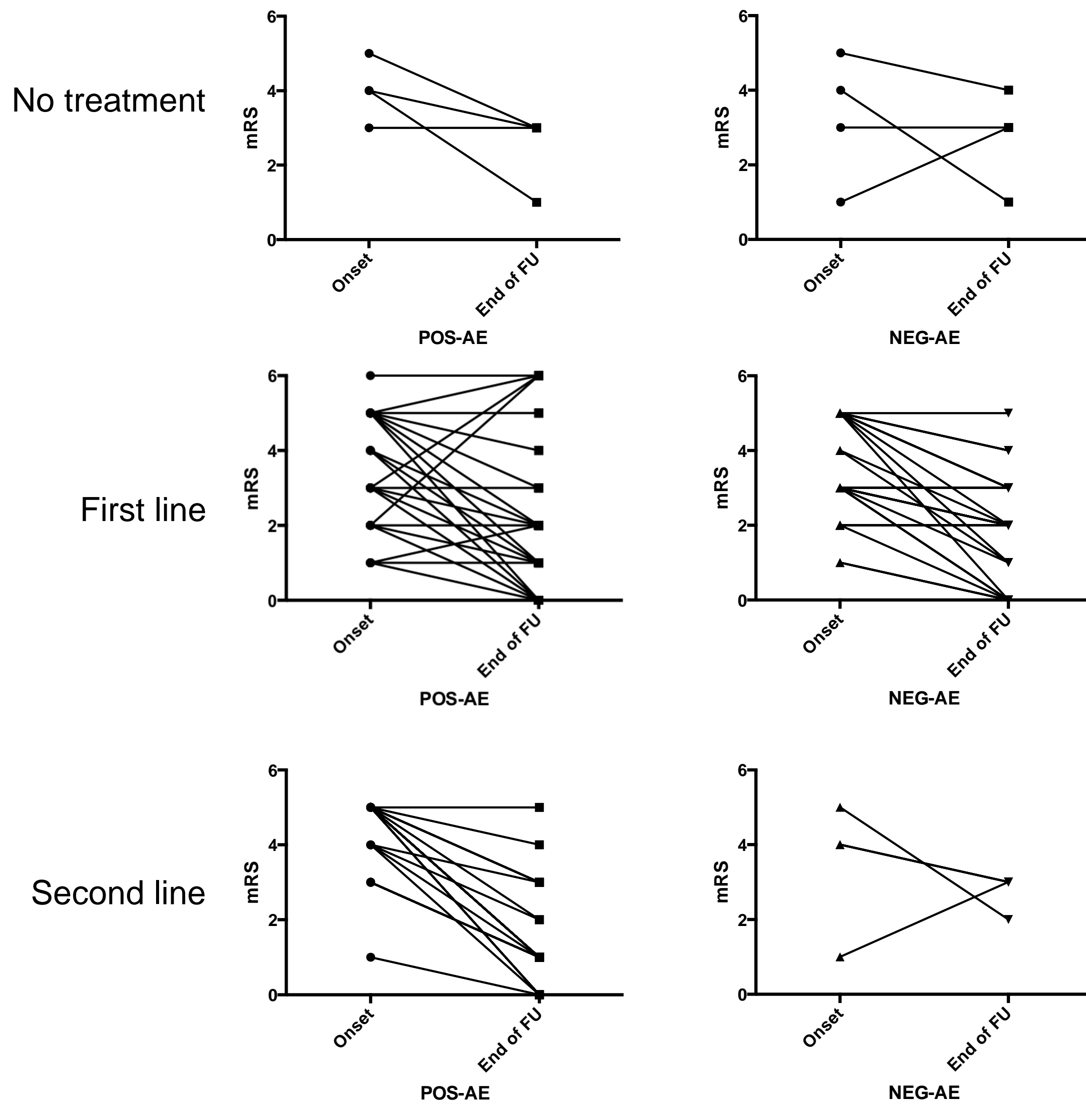
Nineteen patients experienced a relapse, and these were equally distributed between POS-AE and NEG-AE (p=0.57). Median time to relapse was 7 months (range: 1-18). The group where relapses were more frequent was PROB-AE (42.9% of patients) and POS-LE (19.5% of patients). A relapse occurred in only 9.4% of POS-NMDAR. Relapses were more common in patients with inflammatory findings in CSF analysis (p=0.036).

Figure 13: treatments strategies, disease course and outcome in patients with antibody positive and negative autoimmune encephalitis



Patients with POS-AE received more frequently first and second line treatment compared to NEG-AE (A). A relapsing course (B) and poor outcome at the end of follow-up (C) were slightly more frequent in the NEG-AE group, but this did not reach statistical significance. Response to treatment was more frequent in POS-AE patients (D), and in patients treated within 6 months form the onset of the neurological syndrome (E). Patients with POS-AE were more likely to be treated within 6 months (F). AE: autoimmune encephalitis; mRS: modified Rankin scale. Fisher exact test.

Figure 14: disability at disease peak and at the end of follow-up in antibody positive and negative autoimmune encephalitis



The graph represents the modifications of mRS score from disease onset to the end of follow-up for each patient. Clinical course does not differ sensibly between POS-AE and NEG-AE. Concerning patients receiving a second line, a comparison in hard due to the low number of NEG-AE patients treated.

2.3.4 Discussion and conclusions

In this study, we investigated a large retrospective cohort of patients with AE fulfilling the recently established clinical criteria. We identified a group of patients with NEG-AE and explored their clinical and paraclinical profile.

Overall, patients with NEG-AE mostly had a LE phenotype, and did not differ from POS-AE in tumor frequency or disease course. Other than LE, the most frequent clinical phenotype in NEG-AE was seizures and status epilepticus.

A small number of studies investigated the frequency and characteristics of NEG-AE, mainly focusing on NEG-LE.[47-49] In a recent study, Graus and colleagues thoroughly studied a large cohort of patients with LE, identifying 12 that did not show any NAbs.[46] The results from our study are partially discordant. First of all, the proportion of NEG-LE was 29%, much higher to what previously reported (7%). Secondly, the proportion of NEG-LE with cancer was sensibly lower in our study (17.6% vs 42%). There is not a clear explanation for these discrepancies, but it is possible that in patients with NEG-LE that only underwent a first line screening (29% of patients) rarer NAbs could have been missed, leading to patients' misclassification.

As a novelty, we attempted to describe patients with NEG-AE other than NEG-LE. Most patients in this group showed symptoms of limbic system involvement, but could not fulfill the MRI criteria for NEG-LE. Common findings were unilateral temporal lesions, or lesion that were not confined exclusively to the limbic system. Again, patients with PROB-AE and POSS-AE did not differ from POS-AE in relapse rate or tumor frequency.

Considering all patients with NEG-AE, a satisfactory response to treatment was less frequent compared to POS-AE, and outcome was slightly worse, but this could be attributed to two main factors: a) NEG-AE patients were more frequently untreated, and less likely to be treated with second line drugs such as Rtx or CPA; b) NEG-AE patients received a treatment in later stages of the disease.

Taken together, this data support the idea that NEG-AE is a group of disorders comparable to POS-AE, that can respond to immunosuppressive treatments and could benefit from a more aggressive therapeutic approach.

Another relevant finding concerns the laboratory detection of NAbs. Even though most patients were characterized with a first line of screening, our results show that the use of a second line laboratory approach was helpful in identifying

clinically relevant antibodies in over 1/3 of patients with negative NAbs. This is in line with the recommendation from Graus et al. suggesting that, in case of negative results after a first screening, AE samples should be referred to experienced laboratories able to perform second level tests. A more accurate disease classification could lead to a timely diagnosis and treatment initiation, and possibly to a better outcome.

Our study has also limitations. First of all, not all patients included in the NEG-AE group underwent a first and second line of NAbs screening. It is possible, therefore, that some patients were misclassified as NEG-AE. Secondly, the retrospective nature of our data and the heterogeneity of the treatments received do not allow a detailed analysis of the factors associated with treatment response.

In conclusion, we characterized for the first time the group of NEG-AE as a condition with tumor frequency and tendency to relapse similar to POS-AE. In addition, we showed the importance of a second level diagnostic antibody screening for a proper patients classification. Prospective studies are needed to properly assess the optimal treatment strategy for NEG-AE patients.

3 Antibodies against Myelin Oligodendrocyte Glycoprotein in acquired demyelinating disorders of the central nervous system

3.1 General introduction

3.1.1 MOG as an antigenic target in demyelination: from animal models to human diseases

Myelin Oligodendrocyte Glycoprotein (MOG) is a 28.193 kDA protein expressed at the surface of plasma membrane of oligodendrocytes forming the myelin sheets. MOG has always been recognized as exclusively expressed in the CNS myelin, but mRNA expression studies suggested its presence in PNS myelin at ten-fold lower levels.[50] It is a minor component of the myelin sheath, accounting for less than 0.05% of myelin proteins.[51] MOG has 2 transmembrane domains and is part of the immunoglobulin superfamily. The precise function of the protein is unknown, but it is thought to have a role in hemophilic cell adhesion, oligodendrocyte maturation and myelin integrity.[52] In humans, 13 isoforms produced by alternative splicing are known, differing mainly in their cytoplasmic tag. On the cell surface, MOG aggregates in homodimers, but can also create heterodimers with different isoforms.

For its location on the outermost layer of the myelin sheet and its accessibility in-vivo, MOG has often been addressed as a putative antigen in autoimmune demyelinating disorders. In particular, MOG has been studied for years as the most used antigenic inducer and the main antigenic target of encephalitogenic T-cells in experimental autoimmune encephalitis (EAE), a complex animal model of multiple sclerosis (MS).[6] The potential role of MOG antibodies in EAE was firstly explored after the isolation of the MOG monoclonal antibody (mAb) 8-18C5 that was raised against rat cerebellar proteins.[53] The 8-18C5 mAb was able to worsen both relapsing and chronic models of EAE and to contribute to the formation of demyelinating plaques, with an effect that was strictly dependent on the blood-brain-barrier (BBB) damage induced by encephalitogenic T-cells.[53] Importantly, antibodies alone were not able to induce the disease.[54]

It later became clear that antibody responses against MOG target both linear and conformational surface epitopes, and that only the latter had a pathogenic potential.[55] To further complicate the issue, despite a 90% sequence homology between the murine and human isoform of the protein, if has been found that the immunodominant epitopes of MOG antibodies may vary between species, with relevant implication for animal pathogenicity experiments using human antibodies.[56]

Given the experimental background linking MOG antibodies and EAE, many studies investigated the presence of MOG antibodies in patients with MS.

Seminal studies performed using ELISA or western blot for autoantibody detection showed: a) an increased prevalence of MOG antibodies in the serum of patients with MS compared to controls; b) a higher relapse rate in MS MOG antibody-positive patients compared to negative; c) a higher chance of developing MS in patients with a first demyelinating attack and MOG antibodies.[57,58] These studies however had severe limitations mainly connected with the methods used for MOG antibodies detection, and following studies failed to confirm these data.[59,60]

3.1.2 MOG antibodies detected with conformational techniques in human disease

The introduction in clinical practice of more reliable laboratory assay able to detect conformational epitopes provided the basis for a better understanding of the clinical correlates of MOG antibodies, showing that they do not associate with MS, but with other acquired demyelinating syndromes.[61] Initially MOG antibodies were found using an elegant tetramer-based radioimmunoassay in over 40% of patients with ADEM.[62] The presence of MOG antibodies was able to discriminate between MS and ADEM, and antibody titer drop were predictive of a monophasic course.[63] Later studies using a CBA with a full-length protein showed that MOG antibodies can also be found in a percentage of AQP4 antibody- negative patients with neuromyelitis optica spectrum disorders (NMOSD), characterized by the combination of monolateral or bilateral optic neuritis (ON) and myelitis.[64]

3.1.3 Clinical and paraclinical features of MOG associated disease

In the past years, the analysis of large case series of both adult and pediatric patients has allowed a thorough characterization of the disease spectrum associated with MOG antibodies (MOG associated disease, MOGAD), suggesting that it should be considered a separate and unique clinical entity.[65] The most common clinical presentation of MOGAD is ON, that in more than half of the cases is bilateral, and myelitis, that can involve ≥3 metamers (longitudinally extensive transverse myelitis, LETM). Other common presentations include brainstem encephalitis, which can manifest with life threatening symptoms such as central hypoventilation, and ADEM.[66] Up to 15% of MOGAD patients can present with signs of cortical dysfunction such as encephalopathy and seizures, that are usually not found in AQP4 positive NMOSD.[67] Brain MRI is abnormal in at least 1/3 of MOGAD patients and can reveal lesions in corpus callosum, periventricular and juxctacortical area, basal ganglia, thalami and brainstem. Peculiar characteristics that can help to separate MOGAD form MS are low number of lesions (<3), fluffy edges, infratentorial location (in particular periaqueductal or adjacent to the third ventricle, to the area postrema and to the cerebellar peduncles).[68] Such findings, however, might be difficult to distinguish from AQP4-positive NMOSD.[69] Rarer findings include cortical lesions in 16% of patients and leptomeningeal enhancement in 6%.[70] Cerebrospinal fluid (CSF) analysis reveals pleocytosis in 70% of patients, and about 1/3 has a cell count higher than 100 cells/mm^3. Oligoclonal bands (OCB) are less common compared to MS and can be found in 6-15% of patients.[66,69]

MOGAD has been initially considered as a monophasic and relatively benign disease, but increasing data proved that in most cases is highly relapsing and disabling. Over 80% of MOGAD patients undergo a relapse during the disease history, and around half relapse within 2 years.[66,70] Acute attacks usually respond to the administration of steroids or plasma exchange. The risk of relapse is higher in patients not receiving chronic immunosuppression.[69]

3.2 Laboratory detection of MOG antibodies: diagnostic strategies

3.2.1 Rationale and aims of the study

The techniques for the detection of MOG antibodies have been crucial to define the clinical spectrum of MOGAD. Considering the evidence supporting a pathogenic relevance of conformational MOG antibodies in the animal models, CBAs are currently considered the gold standard for their detection.[55] In this technique MOG is expressed in its native and glycosylated form, and the surface location of the protein allows the formation of homodimers, that are likely to increase the autoantibody binding.[71] The use of a human MOG construct is essential to ensure adequate sensitivity and specificity, since the majority of patients harbor antibodies that do not recognize the murine form of MOG.[72] In addition, full-length MOG construct have shown a higher diagnostic sensitivity when compared to truncated MOG forms.[61] Despite the progresses made in the past few years, there are still concerns regarding MOG antibodies specificity, and many authors reported the detection of MOG antibodies in patients with MS even using CBAs.[73,74] In order to address this issue, different strategies have been proposed, that reflect the current heterogeneity in MOG antibody testing: a) a live CBA using IgG1-specific secondary antibodies (LCBA-IgG1), usually testing serum at 1:20 dilution, that should prevent cross reactivity between MOG IgM and a secondary antibody directed to total IgG;[61] b) a live CBA for total IgG with a titration cut-off (generally set at 1:160) obtained through serial endpoint titration of patient's serum;[73] c) a live CBA analyzed through flow-cytometry with a cut off set automatically for each test analyzing in parallel non transfected cells.[75] At present, no systematic study has been performed to compare the diagnostic performance of these tests. Recently, to further complicate this scenario, an easy to perform commercial fixed CBA (CCBA) has been introduced. According to information provided by the manufacturer, it shows good sensitivity and a fair specificity, but systematic data are poor.[76]

Our aim was to implement and compare different laboratory tests, in order to define the best diagnostic strategy for MOG antibody detection.

3.2.2 Methods

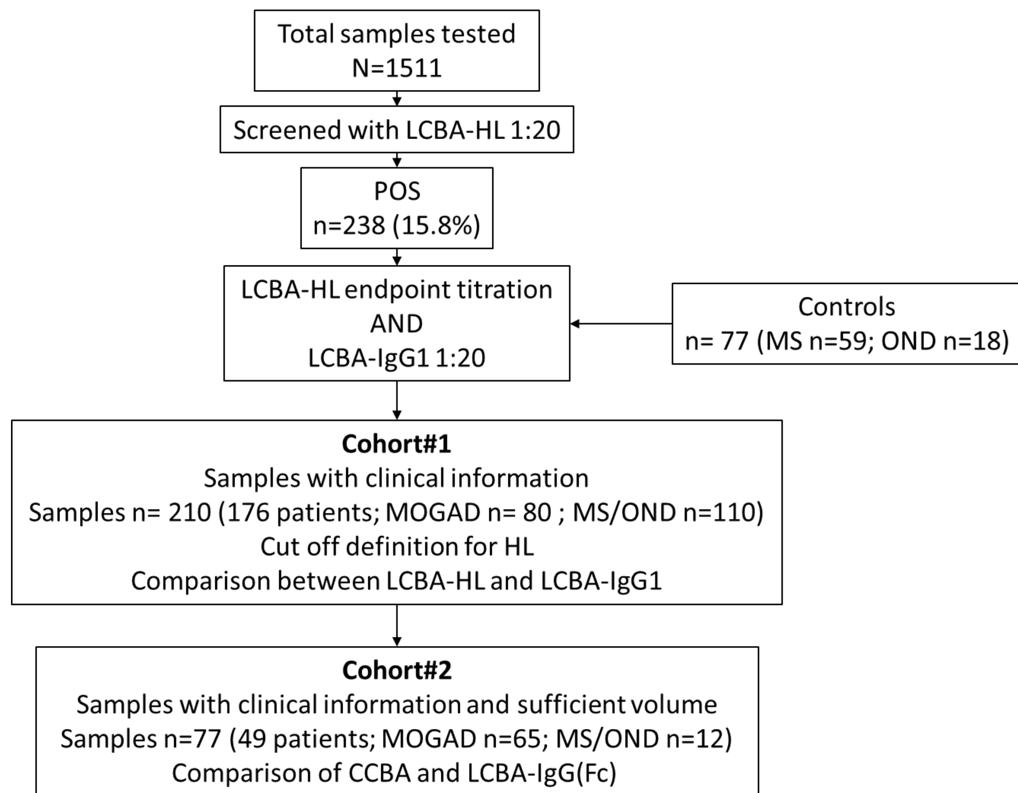
3.2.2.1 Study outline

We prospectively screened 1511 serum samples sent to the Neuroimmunology laboratory of Pavia for MOG antibodies using the routine diagnostic LCBA at 1:20 dilution with a total IgG secondary antibody against the heavy and light chain (LCBA-HL); 238/1511 (15.8%) resulted positive (Figure 15). Seventy-seven controls were additionally tested (59 patients with MS and 18 with dementia). All positive samples were titrated up to their endpoint for total IgG, and in parallel were tested with a live CBA at 1:20 dilution using an IgG1 specific secondary antibody (LCBA-IgG1). All positive samples with clinical information available were used for the assay validation study, along with a group of controls of patients with MS and other neurological disorders (OND) (Cohort #1).

In Cohort #1, the performance of LCBA-HL (best cut off) was compared with the performance of LCBA-IgG1. When discrepant results were found between the two tests and no IgG1 antibodies were identified, samples were tested for IgG2, IgG3, IgG4 and IgM. Samples from this cohort with sufficient volume still available after storage at -20°C were then used for further testing (Cohort#2).

In cohort#2, samples were tested with a live CBA using a total IgG anti-Fc specific secondary antibody (LCBA-Fc) and, when positive, titred up to their endpoint; in addition samples were tested with a CCBA at 1:10 dilution. The performance of LCBA-Fc (best cut off) was then compared with the performance of FCBA, LCBA-HL and LCBA-IgG1.

Figure 15: outline of the study



LCBA: live cell based assay; POS: positive; LCBA-HL: live CBA with total IgG secondary antibody against the heavy and light chain; MS: multiple sclerosis; OND: other neurological disorders; MOGAD: MOG-associated disorders; LCBA-IgG1: live CBA with IgG1 secondary antibody; CCBA: commercial cell based assay; LCBA-Fc: CBA with total IgG secondary antibody against the fragment crystallizable region

3.2.2.2 *Clinical information*

Information for positive samples and controls was collected from outpatient documents and discharge letters. Patients were classified in the following groups:

- MOGAD, if the following criteria were fulfilled:
    - Presence of at least one or a combination of the following clinical syndromes
        - Optic neuritis (mono or bilateral)
        - Transverse myelitis (short or longitudinally extensive)
        - ADEM
        - Brainstem encephalitis
    - Absence of Brain MRI lesions suggestive of MS
    - No evolution to clinically defined MS for at least 1 year
- MS according to the 2017 McDonald criteria[77]
- Other neurological disorders (OND) when not fulfilling criteria for any other group.

Multiple samples from the same patient were labeled with the final clinical diagnosis regardless of the stage of the disease.

For the purpose of this study, patients with MOGAD were considered as having the "true condition", and patients with MS and OND as not having the "true condition".

3.2.2.3 *Laboratory assays:*

LCBAs were performed as described for other antigens at page 25. For the LCBA-HL, a full length MOG construct tagged with emerald fluorescent green protein (EGFP, kindly donated by Dr. Markus Reindl) was used, and patients' MOG antibodies were revealed with a red fluorescent secondary antibody (Alexafluor 564, Invitrogen) diluted at 1:750. For the LCBA-IgG1, an untagged MOG construct was used (kindly donated by Dr. Patrick Waters), and patients' MOG antibodies were revealed with a green fluorescent secondary antibody specific for IgG1 (Alexafluor 488, Invitrogen) diluted at 1:500.

CCBA was performed according to manufacturer instructions, similarly to what reported at page 25.

3.2.2.4 Statistical analysis

Descriptive statistic was performed as reported in other sections (page 45). The receiver Operator Characteristic (ROC) curve was used to define the ideal cut off for LCBA-IgG(HL) and LCBA-Fc. Test performances were evaluated comparing sensitivity, specificity and accuracy.

3.2.3 Results

3.2.3.1 Study population

A total number of 210 samples (corresponding to 176 patients) were included in the study (Cohort#1). Clinical and demographic information are summarized in table 13. Patients were predominantly females (61.4%), and mostly adults (81%). Median follow-time was 22 months (range 2-147). Among 49 patients with MOGAD, the most common clinical syndromes were TM (15/49, 30.6%) and ON (15/49, 30.6%). In this 2 groups, 2/15 patients with ON had a relapsing disease and 2 had simultaneous bilateral involvement, whilst 5/15 patients with TM had a relapsing disease confined to the spinal cord. Ten/49 patients (20.4%) fulfilled the criteria for NMOSD,[64] and 8/49 (16.3%) had ADEM, 7 of which were children.

Table 13: demographic and clinical information of patients from Cohort#1

Patients, n (%)	176 (100)
Male sex, n(%)	68 (38.6)
Age at testing, median(range)	44 (0-90)
Pediatric patients, n (%)	19 (10.4)
Follow-up (months), median (range)	22 (2-147)
Diagnosis	
MOGAD, n (%)	49 (27.8)
TM, n (%)	15/49 (30.6)
ON, n (%)	15/49 (30.6)
NMOSD	10/49 (20.4)
ADEM, n (%)	8/49 (16.3)
Tumefactive brain lesion, n (%)	1/49 (2.0)
MS, n (%)	90 (51.1)
OND, n (%)	37 (21.0)

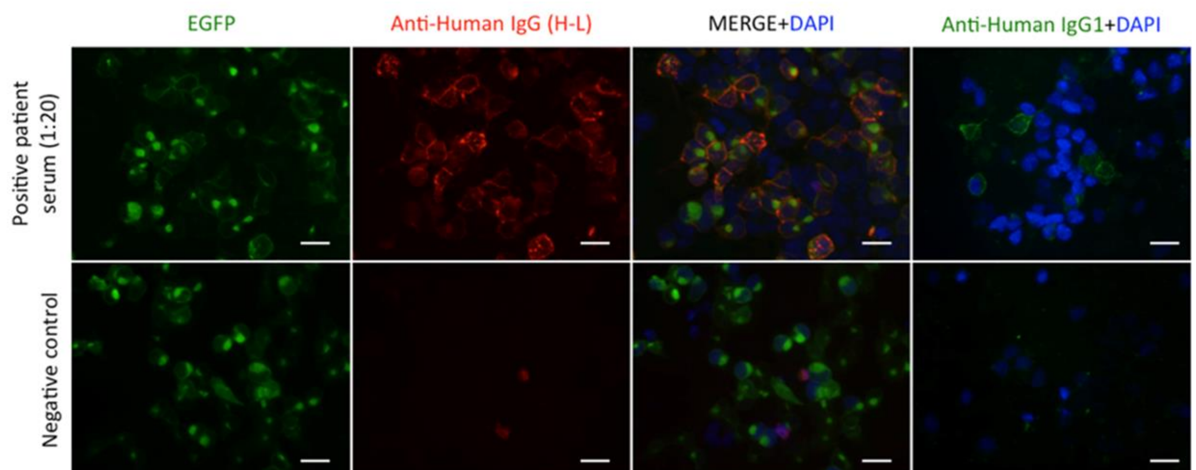
MOGAD: MOG associated disease; TM: transverse myelitis; ON: optic neuritis; NMOSD: neuromyelitis optica spectrum disorder; ADEM: acute disseminated encephalomyelitis; MS: multiple sclerosis; OND: other neurological disorders.

3.2.3.2 Cut off definition for LCBA-HL and comparison with LCBA-IgG1

LCBA-HL at 1:20 dilution was chosen as the screening test.(Figure 16) In order to assess the ideal titration cut-off for this test, a ROC analysis was performed, (Figure 17, A) and a dilution of 1:160 had the best performance.(Table 14) Notably, dilutions equal or higher than 1:640 provided a very high specificity (100%, CI: 97.20-100.00), and were never found in patients with MS or OND.(Figure 17, B) Thirty-one/210 samples (14.76%) had a titer between 1:160 and 1:320, 10/31 with MS/OND and 21/31 with MOGAD.

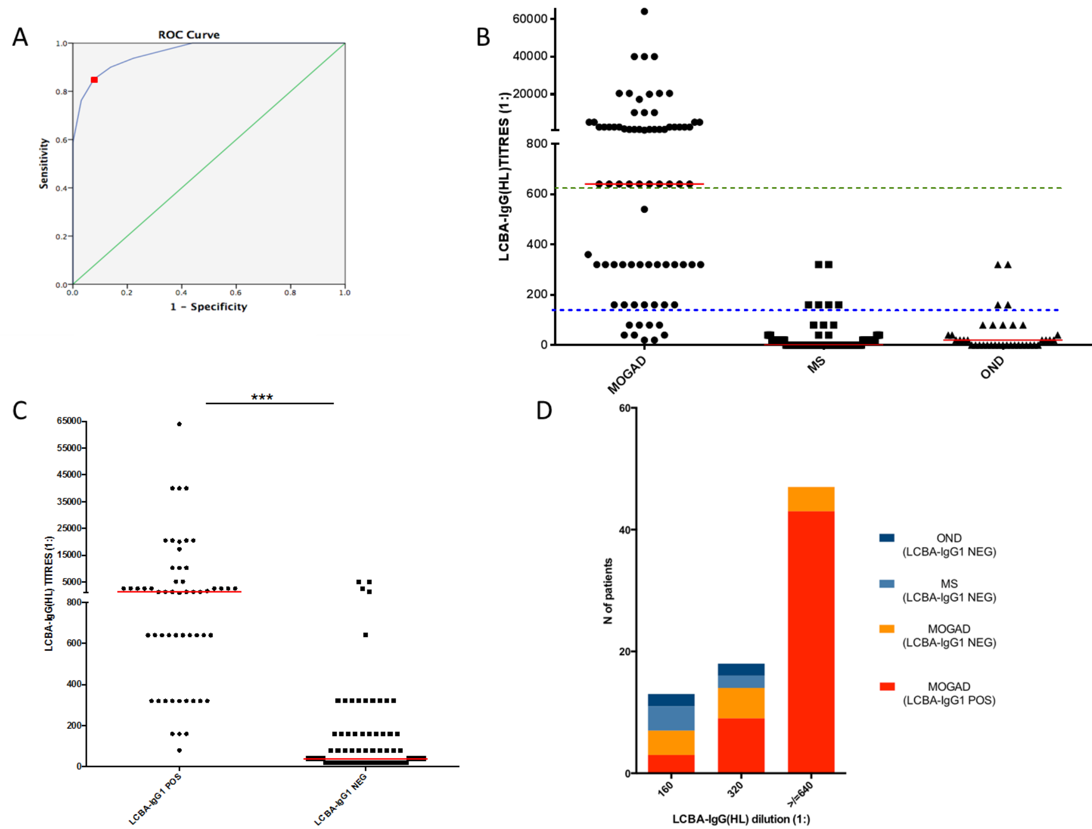
LCBA-IgG1 was highly specific (100%, CI: 93.73-100.00) and was positive only in patients with MOGAD. LCBA-IgG1 was positive more frequently in patients with higher LCBA-HL ($p<0.0001$),(Figure 17, C) and only in 1 sample with a titration lower than 1:160 (1:80). The sensitivity was lower, and the overall accuracy was similar but slightly inferior compared to LCBA-HL (respectively 82.48% vs 83.94%).

Figure 16: LCBA for MOG antibodies



The figure shows results for a patient positive for both LCBA-HL and LCBA-IgG1 (top panels) compared with a negative control (bottom panels). The positive patient shows total IgG binding (red, panels 2-3, top row) to the surface of HEK293 cells transfected with EGFP-tagged MOG (panels 1-3, top row). The binding is confirmed using an IgG1 specific secondary (green, panel 4, top row). The negative control does not provide any staining with both total IgG (panel 2, bottom row) or IgG1 secondary (fourth panel, bottom row). Green: EGFP (panel 1 and 3, top and bottom row) or IgG1 (panel 4, top and bottom row). Red: total IgG (panel 2-3, top and bottom row). Blue: DAPI, which stains nuclei, (panel 3-4, top and bottom row).

Figure 17: Ideal cut off for MOG antibodies and comparison between LCBA-HL and LCBA-IgG1.



A: ROC analysis for the definition of the best cut off for LCBA-HL. The red dot represent the dilution cut off with the best accuracy (1:160); **B**: LCBA-HL titers in MOGAD, MS and OND. The blue dotted line represents the ideal cut-off defined with the ROC analysis (1:160), the green dotted line represents the cut off with 100% specificity (1:640); **C**: LCBA-HL titers in LCBA-IgG1 positive and negative samples. Titers are higher in LCBA-IgG1 positive samples (Mann-Whitney); **D**: clinical groups distribution in different LCBA-HL titrations. The graph represents samples with titres ≥160.

Twenty-three samples (corresponding to 19 patients) with LCBA-HL titer ≥160 were negative when tested with LCBA-IgG1, and clinical and serologic information are reported in table 15.(Figure 17, D) The median titer was 1:320 (range: 1:160-1:5120). Nine/19 patients were classified as MS (n=5) or OND (n=4), all with titers between 1:160 and 1:320. Among the 10 patients with MOGAD and no MOG IgG1, 5 had a TM (in 3 cases relapsing) with negative brain MRI, 2 had ADEM, 1 had monophasic ON and 1 fulfilled the criteria for NMOSD. One patient had a tumefactive demyelinating lesion without OCB on the CSF analysis, and relapsed after steroid suspension. To ascertain the discrepancies between total IgG and IgG1 reactivities, IgG subclasses were tested, and in 7/19 patients other IgG subclasses were identified. IgG2 were found in 4 patients, 3 with MOGAD and 1 with MS, and IgG3 were found in 3 patients, 2 with MOGAD and one with MS. In 9/19 patients, despite repeated testing, no IgG subclasses could be identified; 5/9 had IgM antibodies (3 with MOGAD, 1 with MS and 1 with OND).

Considering the high specificity of LCBA-IgG1 and the high sensitivity of LCBA-HL, we investigated the performance of a combined laboratory strategy. We considered as positive both samples testing positive with LCBA-IgG1 and those with a titration ≥ 640 with LCBA-HL. The combined strategy showed the best diagnostic accuracy compared to LCBA-IgG1 and LCBA-HL alone (respectively 88.28% vs 82.48% and 83.94%) (see table 15).

Table 14: Assay performance for MOG antibody detection in Cohort#1 and #2

Test	Sensitivity% (CI%)	Specificity% (CI%)	Accuracy% (CI%)
Cohort#1			
LCBA-HL	85.00 (75.26-92.00)	82.46 (70.09- 91.25)	83.94 (76.70- 89.65)
LCBA-IgG1	70.00 (58.72- 79.74)	100.00 (93.73- 100.00)	82.48 (75.06- 88.44)
COMB	80.00 (69.17- 88.35)	100.00 (93.28-100.00)	88.28 (81.41-93.29)
Cohort#2			
LCBA-HL	95.38 (87.10% to 99.04)	25.00 (5.49% to 57.19)	84.42 (74.36% to 91.68)
LCBA-IgG1	78.46 (66.51% to 87.69)	100.00 (73.54% to 100.00)	81.82 (71.38% to 89.69)
LCBA-Fc	93.44 (84.05% to 98.18)	72.73 (39.03% to 93.98)	90.28 (80.99% to 96.00)
CCBA	77.05 (64.50% to 86.85)	91.67 (61.52% to 99.79)	79.45 (68.38% to 88.02)
COMB	91.67 (81.61-97.24)	100 (63.03-100)	92.65 (83.67-97.57)

LCBA: live cell based assay; POS: positive; LCBA-HL: live CBA with total IgG secondary antibody against the heavy and light chain; LCBA-Fc: live CBA with total IgG secondary antibody against the fragment crystallizable region; COMB: combined strategy considering both IgG positive and LCBA-HL with titers ≥ 640

Table 15: Clinical information of patients with LCBA-HL positive at ≥1:160 dilution and LCBA-IgG1 negative

Pt n	Sex	Age at first testing (years)	LCBA-HL titer	Other Ig subclasses	LCBA-Fc	CCBA	Final diagnosis	Additional information
1	M	8	5120	IgG2	POS	POS	MOGAD	mTM, negative brain MRI
2	F	46	2560	IgG2	POS	POS	MOGAD	rTM, negative brain MRI
3	F	10	1280	None	POS	NEG	MOGAD	ADEM
4	F	78	640	IgG2	POS	POS	MOGAD	Post-infectious mTM, negative brain MRI
5	F	35	320	IgM	POS	POS	MOGAD	rTM, negative brain MRI, transient OCB
6	M	39	320	None	POS	NEG	MOGAD	Relapsing tumefactive brain lesion, no OCB
7	F	37	320	IgM	NEG	NEG	MOGAD	NMOSD; rON and TM, negative brain MRI
8	F	52	320	IgG3	NEG	POS	MOGAD	rTM, negative brain MRI
9	M	55	160	IgM	NA	NA	MOGAD	ADEM
10	M	10	160	IgG3	POS	POS	MOGAD	mON, negative brain MRI
11	F	20	320	IgG3	NEG	NEG	MS	N.A.
12	F	22	320	None	POS	NEG	MS	N.A.
13	F	42	160	IgG2	NEG	NEG	MS	N.A.
14	F	27	160	NA	NEG	NEG	MS	N.A.
15	F	49	160	IgM	NEG	NEG	MS	N.A.
16	F	69	320	None	POS	NEG	OND	Transient visual loss (likely psychiatric)
17	F	45	320	IgM	NA	NEG	OND	Autoimmune atrophic gastritis with B12 deficiency myelopathy
18	F	51	160	NA	NA	NA	OND	Polyneuropathy
19	F	82y	160	NA	NA	NEG	OND	Dementia

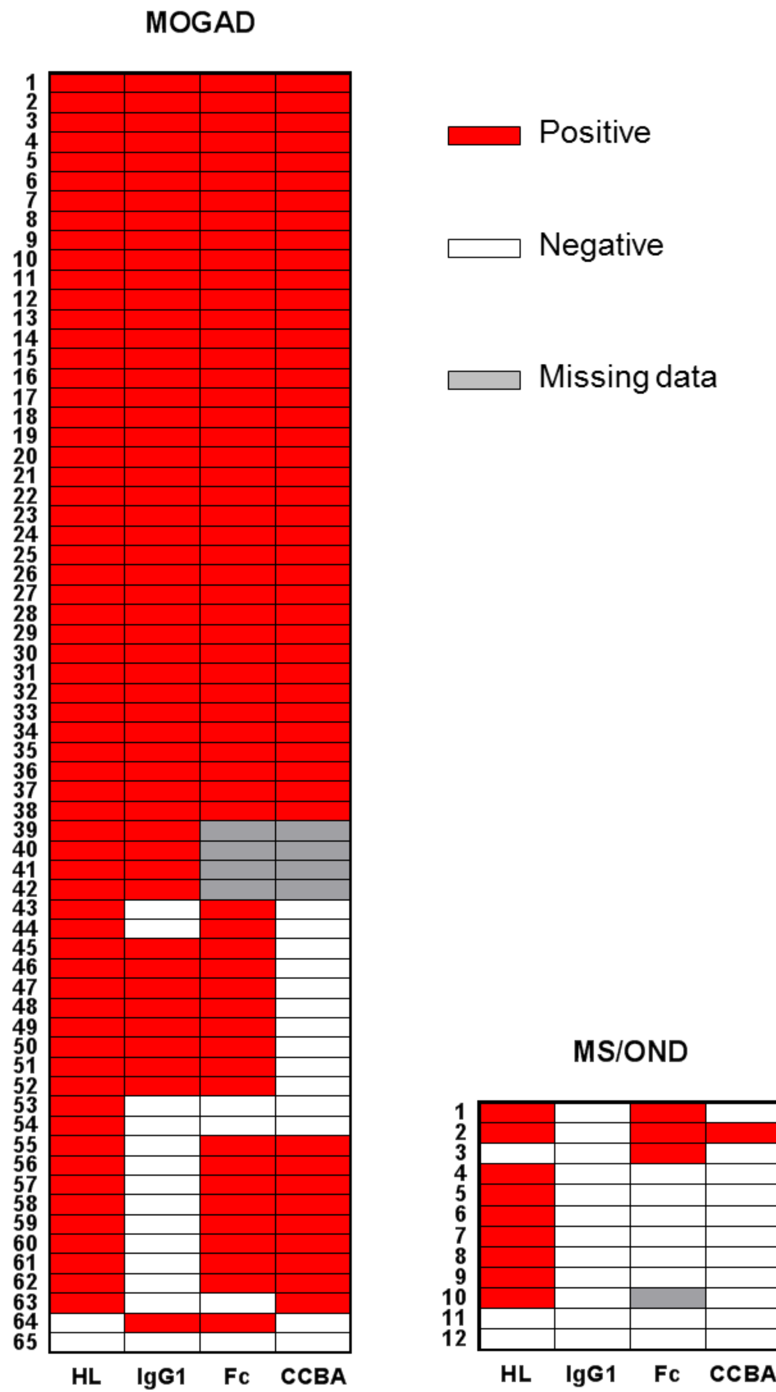
Pt: patient; M: male; F: female; LCBA: live cell based assay; LCBA: live cell based assay; POS: positive; LCBA-HL: live CBA with total IgG secondary antibody against the heavy and light chain; LCBA-Fc: live CBA with total IgG secondary antibody against the fragment crystallizable region; MOGAD: MOG associated disease; TM: transverse myelitis, monophasic (mTM) or relapsing (rTM); ON: optic neuritis, monophasic (mON) or relapsing (rON); ADEM: acute disseminated encephalomyelitis; MRI: magnetic resonance imaging; OCB: oligoclonal bands; NA: not available

3.2.3.3 Global comparison, including LCBA-Fc and CCBA results

A total number of 77 samples previously stored at -20C were defrosted for further testing with LCBA-Fc and CCBA (Cohort#2). Cohort#2 included mostly LCBA-HL positive samples (72/77 samples, 93.5%), and clinical diagnosis were MOGAD in 65/77 (84.4%) and MS/OND in 12/77 (15.6%). CCBA was performed at a dilution of 1:10, according to manufacturer's instructions. For LCBA-Fc endpoint titration were obtained, and a ROC analysis assessed the best performance at the initial screening dilution (1:20). Comparison of the results from the 4 assays is represented in figure 18, and overall assay performance is summarized in table 14. Thirty-three/77 samples had discrepant results between assays, 23 with MOGAD and 10 with MS/OND. As expected from the biased selection of the samples, LCBA-HL was positive in 9/12 samples with MS/OND and had low specificity (25%, CI: 5.49% to 57.19). Fourteen samples with MOGAD were IgG negative, 10/14 LCBA-Fc positive and 9/14 CCBA positive. Interestingly, all MOGAD patients with LCBA-HL titers higher ≥ 640 were LCBA-Fc positive. LCBA-Fc was both sensitive and specific, and had the best overall performance compared with the other tests (accuracy: 90.28%, CI: 80.99-96.00). CCBA had good specificity but failed to identify MOG antibodies in 14/65 MOGAD samples (sensitivity: 77.05%, CI: 64.50-86.85).

The best diagnostic performance was again obtained using a combined strategy (LCBA-IgG1+ LCBA-HL titring ≥ 640) (Accuracy: 92.65, CI: 83.67-97.57).

Figure 18: MOG antibodies assay comparison



The heatmaps show results for different assays from patients with MOGAD (left panel) or MS/OND (right panel). The sample number is reported in the left column.

3.2.4 Discussion and conclusions

In this study, we performed a thorough comparison of the currently available assays for the detection of MOG antibodies, and identified a combination of LCBA-IgG1 and LCBA-HL as the most accurate laboratory strategy.

MOG antibodies have become part of the routine assessment of the acquired demyelinating syndromes, and have been proved to be useful both in discriminating between MS and other demyelinating disorders and in identifying a subset of patients with a highly relapsing disease, that can benefit from early and adequate immunosuppression.[69] Treatments that can be beneficial in MS can be ineffective or harmful in MOGAD, making patients misclassification particularly problematic.[78] Therefore, laboratory detection of MOG antibodies is of the utmost importance, and high accuracy is essential. Assay comparison studies in this field are challenging for 2 main reasons: a) the diseases of the spectrum are rare, b) there is no diagnostic gold standard nor definite diagnostic criteria for MOGAD that could help to define the "true condition". In our study we performed a comparison of the main assays available for MOG antibody detection, willingly using a wide clinical definition for MOGAD, which is derived from studies that define the disorder using the presence of the antibody itself. Since sensitivity and specificity of MOG antibody testing have not been fully validated, misclassification of patients is still likely, and these shortcomings could affect our results too.

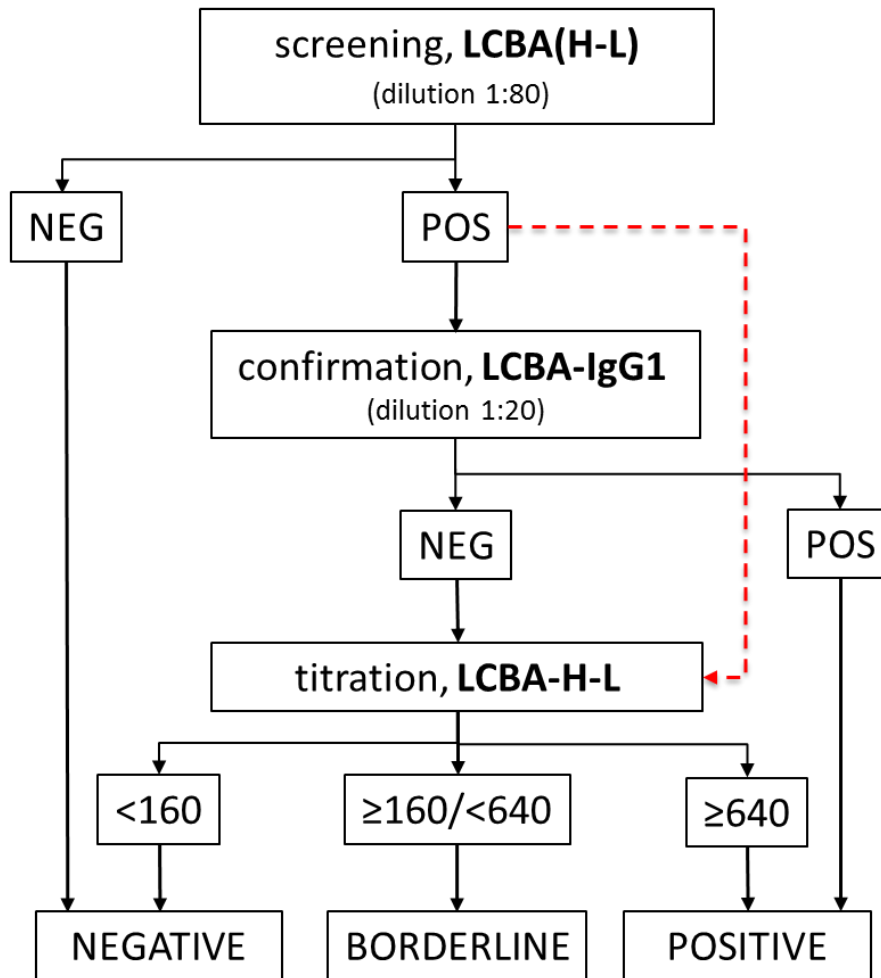
In our hands, each test for MOG antibody detection showed a relatively good performance, and 89.05% of samples in cohort#1 provided concordant results. LCBA-HL had a slightly better performance compared to LCBA-IgG1, even though it was less specific. LCBA-IgG1 was, on the other hand, the most specific assay.
LCBA-HL performance was strictly dependent on titers, with a 100% specificity for titers ≥ 640. This finding facilitates interpretation of the laboratory results, and helps providing the most informative reports for the clinicians.

The CCBA, being widely available and easy to perform, will probably become the most commonly used testing method. In our hands, this test had lower sensitivity and was the single worst performing assay, even though the accuracy was very close to LCBAs. Laboratories relying on this assay should be aware of the risk of false negative results.

We showed that, in order to improve the diagnostic performance, a combination strategy was needed. The association of the most sensitive assay (LCBA-HL) and of the most specific assay (LCBA-IgG1) provided the best diagnostic accuracy, with an increase of 4-6 % in cohort#1 and 8-11% in cohort #2 compared to their independent use. In cohort#2, LCBA-Fc was the single best performing assay, but its use in a combined strategy did not lead to any improvement in accuracy. This improvement is particularly relevant considering the impact that MOG antibody result can have in patients management and, according to our data, we established a multi-step approach for MOG antibody detection in order to provide the most informative results (Figure 19).

In conclusion, our data provide practical information for assay results interpretation, as they are now available from the current literature, and propose an efficient laboratory strategy that maximizes the accuracy. Laboratories that rely on single tests should be aware of the limitations of each assay. With positive results associated with a doubtful clinical condition, or negative results associated with a highly suggestive clinical condition, such laboratories should consider a referral to second level laboratories that use live cells and are able to perform a combined approach.

Figure 19: a multistep diagnostic strategy for MOG antibody detection



Serum samples from patient with suspect MOGAD can be screened using a live cell based assay with a total IgG secondary against heavy and light chains (LCBA-HL) 1t 1:80 dilution; positive samples will undergo confirmation with a LCBA with an IgG1 specific secondary antibody (LCBA-IgG1). Negative samples will undergo a second confirmation step, and will be titrated with LCBA-HL. Titres ≥640 will be reported as positive. Since false positives re more likely between 1:160 and 1:320 dilutions, such results will be reported as borderline. Alternatively, if an IgG1 secondary is not available, laboratories could directly perform the titration step (red dotted arrow).

3.3 MOG antibodies in acquired demyelinating disorders: clinical profile in adults and pediatric patients and relevance of antibody titers

3.3.1 Rationale and aims of the study

Patients with MOG antibody-associated disorders (MOGAD) can have different clinical presentations that include optic neuritis (ON), transverse myelitis (TM), neuromyelitis optica spectrum disorders (NMOSD) and ADEM. Some groups still report the presence of MOG antibodies in a minority of patients with MS, especially in pediatric cohorts, but it is unclear whether this could be attributed to limitations of the laboratory techniques.[79] The clinical presentation seems to be dependent on the age of the patients, with ADEM being more frequent in the pediatric population.[80]

An open debate concerns the utility of MOG antibody titers as prognostic biomarkers, and whether follow-up testing could be helpful to clinicians to orient therapeutic decisions and monitor response to treatments.[65] Preliminary data suggest that persistence of the antibodies over time can associate with a relapsing course.[69] However, these data need conformation on independent and large series of patients, and precise indication regarding timing of retesting is still lacking.

Within a multi-center study, we aimed to characterize a large cohort of patients with suspected MOGAD, whose MOG antibody testing was centralized in a single laboratory, and to define the role of longitudinal antibody testing in clinical practice.

3.3.2 Methods

3.3.2.1 Patients and samples

Sera of 1241 patients with possible acquired demyelinating syndrome (ADS) were prospectively tested from June 2016 to January 2018 for the presence of MOG antibodies and AQP4 antibodies. MOG antibody testing was performed using a LCBA with a multi-step confirmation strategy (see section 3.2). AQP4 antibodies were measured with a commercial CBA (Euroimmun, Lübeck). Only patients with sufficient information available and at least 3 months of follow-up were included in the study. Patients <16 years old were considered pediatric. Clinical information was collected from discharge letters and outpatient clinic reports. Disability was measured at the peak of the neurological syndrome using the expanded disability status scale (EDSS), and severe disability was defined as EDSS score ≥ 3. A clinical presentation was labeled as "encephalitis" if cortical signs such as encephalopathy or seizures were present. A relapse was defined as a new inflammatory event occurring at least 1 month after the resolution of the previous episode. Patients were classified at the end of follow-up on the basis of their neurological manifestations. NMOSD was defined according to Wingerchuck criteria.[64]

All samples obtained from MOG positive patients were titred up to their endpoint using the LCBA-IgG(HL). Samples were considered to be from the acute phase when they were obtained within 3 months from the onset of the neurological disease.

To investigate the longitudinal dynamics of antibody titers, we considered patients with at least 2 samples available taken 6 months apart. For titers dynamic correlation studies, we considered patients with an available sample from the acute phase and one taken during remission, defined as the absence of disease for at least 3 months. When multiple remission samples were available we selected the sample with the lowest titer.

3.3.2.2 Statistical analysis

Descriptive statistic was performed as described in other sections (see page 45). Paired longitudinal titrations were analyzed with the Wilcoxon matched paired sum test.

3.3.3 Results

3.3.3.1 Population characteristic and comparison between pediatrics and adult patients

Among 1241 patients tested, 36 were positive for MOG antibodies (2.9%), but one was excluded for insufficient clinical information. Clinical and paraclinical information of MOG antibody positive patients are summarized in table 17. Twenty-two/35 patients were adults (62.9%) and 13 were pediatrics (37.1%). Median age was 40 years (range: 17-78) for the adult group and 8 years (range: 2-12) for the pediatric group. Fourteen/35 patients (40%) were males. Median follow-up time was 55 months (range: 5-181). At the end of follow-up, patients were classified in the following clinical groups: NMOSD (11 patients, 31.4%), ON (10 patients, 28.6%), TM (6 patients, 17.1%, 4 relapsing) and ADEM (7/35, 22.0%). Three patients with ADEM had relapsing demyelinating attacks, in 2 cases an ADEM and in 1 case an episode of TM (multiphasic ADEM). Finally, one patient with ON that relapsed with a cerebellar demyelinating lesion and no OCB was classified as “other demyelinating disorder”. None of the patients developed MS during follow-up.

The most common clinical presentation was ON (17/35 patients, 48.6%), followed by TM (8/35, 22.9%) and encephalitis (6/35, 17.1%). A bilateral ON at onset was found in 5/17 patients with ON (29.4%), and retrobulbar pain was reported in only 9/17 patients (52.9%). Other less common presentations included a combination of TM+ON (3 patients, 8.6%), and brainstem encephalitis (1 patient, 2.9%). Clinical presentation differed according to age. TM, or a combination of ON+TM, were found mostly in adult patients, whilst an encephalitic onset was found mainly in pediatric patients (38.5% vs 4.5%, p=0.04).

EDSS during the acute phase was available for 29 patients, and 14/29 showed an EDSS score ≥ 3 (40%, median EDSS: 2.5, range: 1-6.5).

All patients underwent a brain and spinal cord MRI. Eighteen/35 patients had an altered brain MRI (51.4%). Multifocal lesions were present in 13/18 patients (72.2%). Lesions location was diverse and included the periependimal areas in the brainstem (8/18, 44.4%), the thalamus (5/18, 27.8%) and the cerebellar peduncle (4/18, 22.2%). Sovratentorial brain lesions in locations suggestive of MS (juxtacortical, periventricular or corpus callosum) were found in 12/18 patients

(66.7%). Juxtacortical lesions were found more frequently in children compared to adults (66.7% vs 11.1%, p=0.05). Contrast enhancing lesions were present in less than half of the patients (8/18, 44.4%). Optic nerve showed swelling in 10/20 patients with ON involvement (50%), and in most cases with contrast enhancement (9/10, 90%). Three/10 (30%) patients had posterior lesions involving the optic chiasm.
Spinal cord MRI was altered in 17/35 patients (48.6%), an in half of them lesions spanned for >3 metamers (8/17, 47%).

Information on treatment were not available for 2 patients. During the acute phase all the remaining patients received a treatment, in most cases with IvMP (32/33 patients, 97.1%). Two patients underwent additional treatment with PlEx (n=1) and with IvIg (n=1). Fifteen/33 patients underwent maintenance therapy with oral steroids for at least 3 months. During follow-up, 6/15 received additional immunosuppression (Rituximab n=3 patients, Rituximab + Tocilizumab n=1 patient, cyclophosphamide + methotrexate n=1 patient, azathioprine n=1 patient). Two patients were initially misdiagnosed as MS, and received accordingly a treatment with dimethylfumarate and interferon-1b. Both patients eventually experienced relapses. Recovery form the acute attack was partial in 18/35 patients (51.4%).

Relapses were found in 19/35 patients (54.3%), similarly in adults and children. Median time to relapse was 7 months (range: 1-178). Notably, in 2 patients relapses occurred respectively 10 and 14 years after the first demyelinating event. Relapses involved the same district as the first demyelinating event in 11/19 patients (57.9%).

Table 17: features in adult and pediatric patients with MOG antibodies

Patients, n (%)	All, 35 (100)	Adult, 22 (100)	Pediatric, 13 (100)	p-value
Male sex, n (%)	14 (40.0)	8 (36.4)	6 (46.2)	0.72
Age at onset (years), median (range)	29 (2-78)	40 (17-78)	8 (2-12)	<0.001*
Months of follow-up, median (range)	55 (5-181)	85 (12-181)	17 (5-155)	0.36*
Prodromal event, n (%)	13 (37.1)	5 (22.7)	8 (61.5)	0.03
Presentation				
ON, n (%)	17 (48.6)	10 (45.5)	7 (53.8)	0.04
TM, n (%)	8 (22.9)	7 (31.8)	1 (7.7)	
ON+TM, n (%)	3 (8.6)	3 (13.6)	0 (0.0)	
Brainstem, n (%)	1 (2.9)	1 (4.5)	0 (0.0)	
Encephalitis, n (%)	6 (17.1)	1 (4.5)	5 (38.5)	
Symptoms				
motor, n (%)	7 (20.0)	4 (18.2)	3 (23.1)	1.00
sensory, n (%)	13 (37.1)	11 (50.0)	2 (15.4)	0.04
sphyncteric, n (%)	11 (31.4)	8 (36.4)	3 (23.1)	0.48
encephalopathy, n (%)	6 (17.1)	1 (4.5)	5 (38.5)	0.01
visual impairment, n (%)	21 (60.0)	13 (59.1)	8 (61.5)	1.00
retrobulbar pain, n (%)	9 (25.7)	6 (27.3)	2 (23.1)	0.78
EDSS ≥3 at onset, n (%)	14/29 (48.3)	8/21 (38.1)	6/8 (75.0)	0.11
LP performed, n (%)	32 (91.4)	19/22 (86.4)	13/13 (100.0)	
CSF cells, median (range)	6 (0-165)	6.5 (0-136)	2 (1-165)	0.40*
OCB, n (%)	6 (17.1)	5 (22.7)	1 (7.7)	0.38
Altered brain MRI, n (%)	18 (51.4)	9 (42.9)	9 (69.2)	0.17
Iuxtacortical, n (%)	7/18 (38.9)	1/9 (11.1)	6/9 (66.7)	0.05
Periventricular , n (%)	2/18 (11.1)	1/9 (11.1)	1/9 (11.1)	1.00
Corpus callosum, n (%)	3/18 (16.7)	2/9 (22.2)	1/9 (11.1)	1.00
Periependimal, n (%)	8/18 (44.4)	5/9 (55.6)	3/9 (32.9)	1.00
Cerebellar peduncle, n (%)	4/18 (22.2)	3/9 (33.3)	1/9 (11.1)	0.26
Thalamus, n (%)	5 (27.8)	1 (11.1)	4 (44.4)	0.29
Contrast enhancement, n (%)	8/18 (44.4)	3/18 (33.3)	5/18 (55.6)	0.64
Optic nerve MRI altered, n (%)	10 (28.6)	3 (13.6)	7 (58.8)	0.02
Chiasmatic lesions, n (%)	3/10 (30.0)	2/3 (66.7)	1/7 (14.3)	0.10
Contrast enhancement, n (%)	9/10 (90%)	2/3 (66.7)	7/7 (100)	0.30
Spinal cord MRI altered, n (%)	17 (48.6)	13 (59.1)	4 (30.8)	0.11
LETM, n (%)	8/17 (47.1)	6/14 (46.2)	2/3 (50.0)	1.00
Contrast enhancement, n (%)	6/17 (35.3)	6/14 (42.9)	0/3 (0.0)	0.52
Attack recovery				
Partial, n (%)	18 (51.4)	14 (60.9)	4 (33.3)	0.16
Complete, n (%)	17 (48.6)	9 (39.1)	8 (66.7)	
Relapses, n (%)	19 (54.3)	12 (54.5)	7 (53.8)	1.00
Months to relapse, median (range)	5 (1-178)	8.5 (1-178)	4 (1-23)	0.55*
Relapse in the same district as the first episode, n (%)	11/19 (57.9)	8/13 (61.5)	3/6 (50.0)	1.00
Treatments				
Oral steroids, n (%)	18/33 (51.4)	10/22 (45.5)	8/11 (72.7)	0.27
IvMP, n (%)	32/33 (97.0)	22/22 (100)	10/11 (90.9)	0.33
IvIG, n (%)	2/33 (5.7)	1/22 (4.5)	1/11 (9.1)	1.00
PlEx, n (%)	1/33 (2.9)	1/22 (4.5)	0/11 (0.0)	1.00
Azathioprine, n (%)	2/33 (5.7)	1/22 (4.5)	1/11 (9.1)	1.00
Cyclophosphamide, n (%)	1/33 (2.9)	1/22 (4.5)	0/11 (0.0)	1.00
Rituximab, n (%)	3/33 (9.1)	3/22 (13.6)	0/11 (0.0)	0.53
Final diagnosis				
mON, n (%)	10 (28.6)	5 (22.7)	5 (38.5)	0.01
mTM, n (%)	2 (5.7)	2 (9.1)	0 (0.0)	
rTM, n (%)	4 (11.4)	4 (18.2)	0 (0.0)	
NMOSD, n (%)	11 (31.4)	10 (45.5)	1 (7.7)	
ADEM, n (%)	7 (20.0)	1 (4.5)	6 (46.2)	
Other demyelinating disorder	1 (2.9)	0 (0.0)	1 (7.7)	

MOG: myelin oligodendrocyte glycoprotein; ON: optic neuritis; TM: transverse myelitis; ADEM: acute disseminated encephalomyelitis; EDSS: expanded disability status scale; LP: lumbar puncture; CSF: cerebrospinal fluid; OCB: oligoclonal bands; MRI: magnetic resonance imaging; LETM: longitudinally extensive transverse myelitis; IvMP: intravenous methylprednisolone; IvIg: intravenous immunoglobulin; PlEx: plasma exchange; mON: monophasic ON; mTM: monophasic TM; rTM: relapsing TM; NMOSD: neuromyelitis optica spectrum disorder; *: Mann-Whitney test

3.3.3.2 Patients with peripheral nerve involvement

Three/35 patients (8.6%) had an involvement of the peripheral nervous system (PNS) manifesting as spinal roots enhancement in lumbosacral MRI (Table 18). All patients were adults, and had high disability during the acute phase of the disease. In addition, all patients had a spinal cord involvement, in 1 case in the setting of an ADEM. Two/3 patients had a relapsing course. All patients with PNS involvement had inflammatory signs at the CSF analysis, including OCB, that were consistently more frequent compared to patients without PNS involvement (p=0.002).

Table 18: patients with MOG antibodies and peripheral nerve involvement

Pt n	Sex	Age at onset	MOG ab titre	Clinical syndrome at onset	PNS involvement	EDSS at disease peak	Brain MRI	CSF analysis	Disease course	Treatment	Final diagnosis
1	F	78y	1:640 (IgG3)	TM	MRI: Spinal roots enhancement ENG: not altered	5		12 LMN, OCB	Monophasic	IvMP, Pr	TM
2	F	40y	1:64000 (IgG1)	ON	MRI: Spinal roots enhancement ENG: not performed	6.5	Negative	93 LMN/mm3, OCB	Relapsing (14 years after onset a second episode of ON; 2 years later TM with PNS involvment	IvMP, Rtx	NMOSD
3	M	52y	1:640 (IgG1)	Encephalopathy with focal neurological signs	MRI: Spinal roots enhancement ENG: not altered	4	Multiple lesions involving grey and white matter Gad+	6 LMN/mm3, OCB	Relapsing (7 months after onset episode of TM)	IvMP, PlEx	Multiphasic ADEM

MOG: myelin oligodendrocyte glycoprotein; PNS: peripheral nervous system; TM: transverse myelitis; EDSS: expanded disability status scale; CSF: cerebrospinal fluid; LMN: lymphmonocytes; OCB: oligoclonal bands; MRI: magnetic resonance imaging; ENG: electroneurography; IvMP: intravenous methylprednisolone; IvIg: intravenous immunoglobulin; PlEx: plasma exchange; NMOSD: neuromyelitis optica spectrum disorder; ADEM: acute disseminated encephalomyelitis;

3.3.3.3 MOG antibody titers

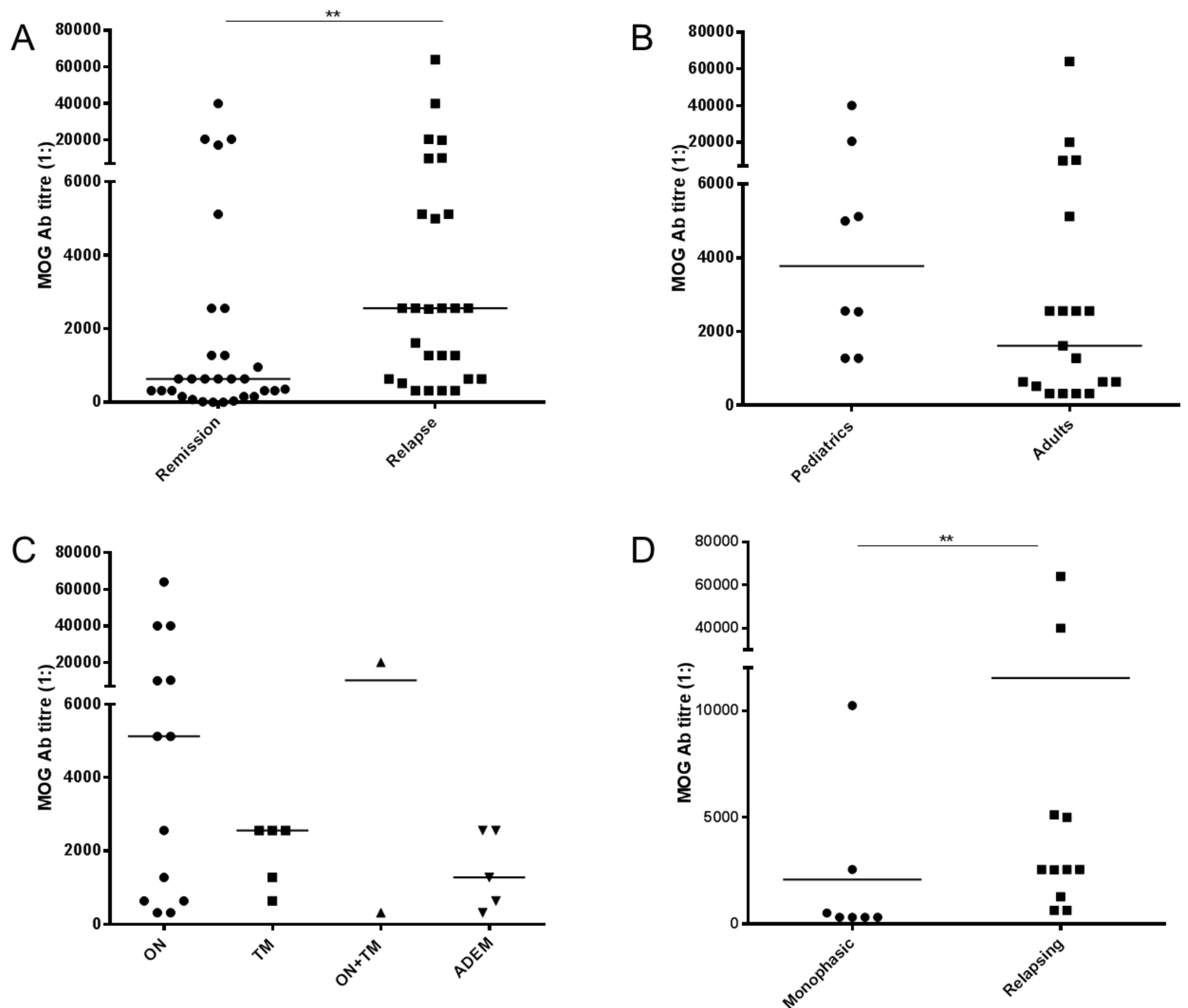
We then tried to define the clinical relevance of MOG antibody titers. Titers from all the samples available from the 35 patients of the cohort were analyzed, and labelled accordingly to the timing of serum collection. Two samples were excluded because obtained shortly after treatment. A total number of 57 samples were studied, 30 taken during disease remission and 27 during the acute phase. Median titer was 1:960 (range: 0-64000). Median titers were higher during relapses (median titer: 1: 2560, range: 320-64000) compared to remission phases (median titer: 640, range: 0-40000,figure 20, panel A, p=0.004). In addition, titers in the acute phase were higher in pediatric patients (median: 1:3780, range: 1280-40000) compared with adults (median 1620, range: 320-64000), even though this did not reach statistical significance (Figure 20, panel B, p=0.15). No significant differences in titers was detected in different clinical presentations (Figure 20, panel C, p=0.57). In order to assess whether titers at onset were able to predict the disease course, we then selected 18 samples collected from the initial demyelinating episode (before any treatment was given) in patients with at least 2 years of follow-up. Interestingly, higher titers correlated with a relapsing course compared to monophasic (Figure 20, panel D, p=0.03).

We then studied the longitudinal evolution of MOG antibody titers. Fourteen patients had two or more samples taken at least 6 months apart. Median time from the first to the last sample was 20.5 months (range: 6-139).Overall, a slight trend toward titer decline was found in monophasic (p=0.14) versus relapsing patients (p=1.00), but this did not reach statistical significance (Figure 21). In 9/14 the first sample available was from the first demyelinating event. Titers dropped below the cut-off (1:160) in 4/8 patients with monophasic disease and in only 1/6 with relapsing disease (that was treated with chronic immunosuppressive therapy). Among these patients, antibodies became undetectable only in 2/5 patients, both with monophasic disease. Titers remained above the cut-off in 8 patients, in most cases with a consistent drop during the remission phase, and half of them relapsed (Figure 22 and 23). In only 2 patients titers remained above 1:5000 during the whole follow-up, one with highly active relapsing disease treated with both rituximab and tocilizumab, and one with monophasic TM. Notably, in the last

patient (Figure 23, panel G), MOG antibodies persisted at high titers (1:2560) for over 11 years, with no signs of relapses and negative follow-up MRI studies. Surprisingly, two patients had MOG antibodies below the cut-off during the acute phase (one with relapsing ADEM and one with monophasic ON) that increased during the remission phase (Figure 22, A; figure 23, D).

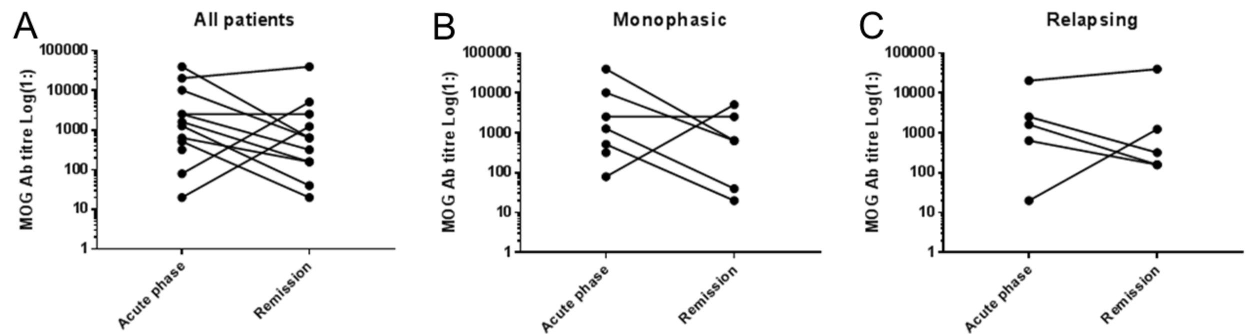
In one patient with bilateral ON and one lesion in corpus callosum samples were prospectively collected every 3 months (Figure 23, panel F). After the first demyelinating attack the patient received IvMP and oral steroids, with complete resolution of symptoms and a decrease of MOG antibodies titer from 1:1240 to 1:160. After 3 months without therapy, titers started to increase again. Once the titers surpassed 1:2000, the patient was preventively treated with a course of IvMP, but two weeks later she developed a bilateral ON. She was then treated with plasma exchange and rituximab, with recovery and a reduction of antibody titers.

Figure 20: MOG antibody titers according to age at onset, disease phase and disease course



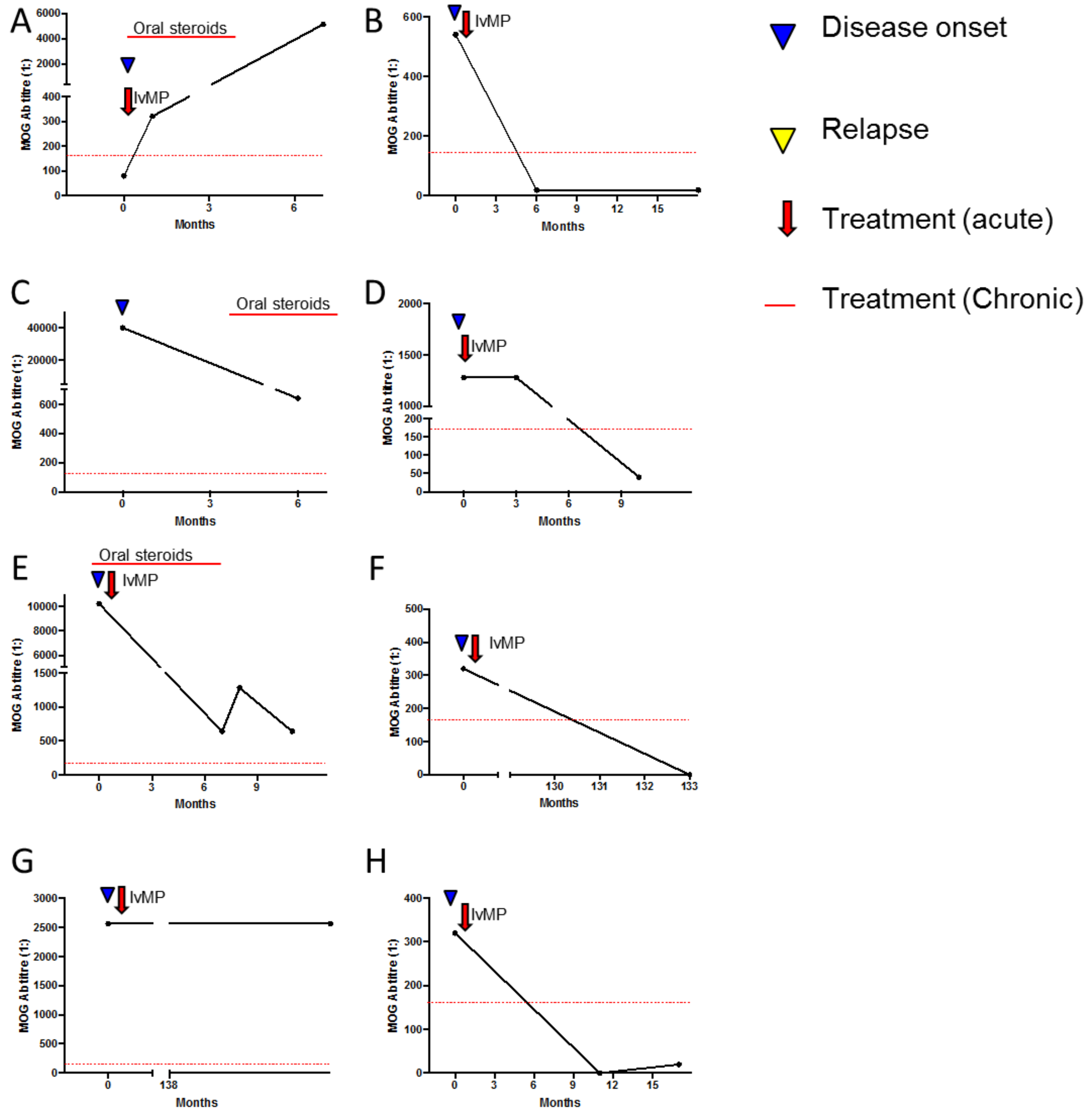
MOG antibody titers were higher during relapse phases compared to remission (A). Considering only samples collected during the acute phase, titers were higher in pediatric patients (B). No significant differences between titers were found according to clinical presentation. In patients with at least 2 year of follow-up, higher titers correlated with a relapsing course (D). MOG: myelin oligodendrocyte glycoprotein; ON: optic neuritis; TM: transverse myelitis; ADEM: acute disseminated encephalomyelitis.

Figure 21: MOG antibody titers dynamics in monophasic and relapsing patients



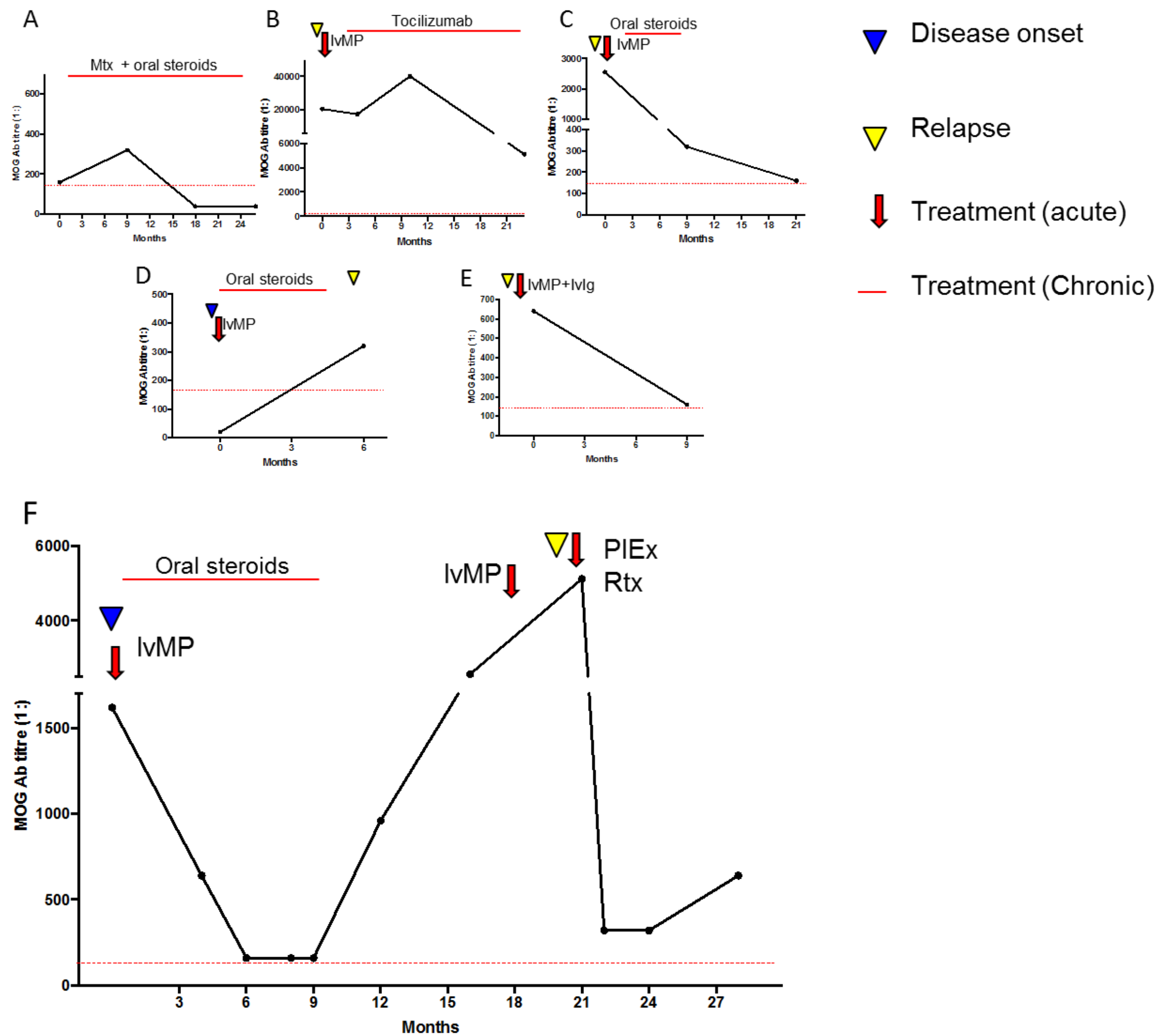
A slight trend toward decline of titre from acute phase to remission was found in monophasic patients, but this did not reach statistical significance (p=0.14 vs p=1.00, Wilcoxon matched paired sum test)

Figure 22: MOG antibodies longitudinal analysis in patients with monophasic disease



In 3/8 patients with monophasic disease antibodies were persistently positive during follow-up (C,E,G). In one patients antibodies were initially below the cut-off and increased during remission. In the remaining 4 patients MOG antibody titers dropped below the cut-off (and in 1 case became undetectable). MOG: myelin oligodendrocyte glycoprotein; IvMP: intravenous methylprednisolone.

Figure 23: MOG antibodies longitudinal analysis in patients with relapsing disease



In 5/6 patients with relapsing disease titers remained above the cut-off, in most cases with a consistent drop (B-F). In one patient titers dropped below the cut-off, but this happened during chronic immunosuppressive therapy (A). In one patient with ADEM titres were below the cut-off at onset and increased during follow-up (D). In patient (F) the increase of antibody titres predicted a clinical relapse. MOG: myelin oligodendrocyte glycoprotein; IvMP: intravenous methylprednisolone. PlEx: plasma exchange; Rtx: rituximab.

3.3.4 Discussion and conclusions

In this study we characterized a large cohort of adult and pediatric patients with MOG antibodies, and we investigated the role of antibody titers in clinical management.
In line with other studies, we found that MOG titers are higher during the acute phase compared to relapse and tend to be higher in children compared to adults. In a recent paper, titers >1:2560 were always associated with relapses.[66] We did not confirm such finding, and, accordingly, were not able to identify a clear cut-off to separate relapse and remission. Ultimately, antibody titer dynamics was extremely variable between patients.

We confirmed that MOGAD patients are at high risk of developing relapses, that we found in over 50% of the patients and with equal distribution between children and adults.[66] As a novelty, we found a correlation between higher titers at presentation and the occurrence of relapses during follow-up. This correlation was not found in other studies, possibly because patients with shorter follow-up were included in the analysis. If findings will be confirmed in larger prospective studies, they could provide essential information to stratify the relapse risk in MOGAD patients. Notably, in our cohort, relapses occurred in very large time span, and 2 patients relapsed over 10 years after the first demyelinating episode. This is in line with other studies, reporting a relapse rate of up to 93% in patients with long follow-up, and supports the use of chronic immunosuppression in these patients.[78]

The analysis of longitudinal titers in our patients largely reflects what emerged in other studies, where a persistence of the antibodies was associated with a higher relapse risk.[69,78,81] Indeed, in our cohort, none of the patients where antibodies became negative developed relapses during the follow-up, except for one undergoing chronic immunosuppressive therapy. The persistence of MOG antibodies, however, was not always associated with a relapsing course, and in one patient high titers persisted for over 11 years with no disease activity. This suggests that, similarly to AQP4 mediated disease, antibodies alone, even at high titers, are not sufficient to cause the disease. Additional yet unexplored mechanisms possibly associated with local blood brain barrier dysfunction and/or T-cell activation are likely to be needed for the antibodies to reach their target in the central nervous system and cause demyelination.

In one patient the rising of antibody titers over time anticipated a relapse, that occurred despite the administration of preventive treatment. The close correlation between MOG titers and disease activity on one hand supports the pathogenicity of the antibodies, and on the other hand suggest the usefulness of regular titrations in clinical practice. These findings, though derived from a single case, suggest that patients at the initial stage of the disease, or for which chronic immunosuppression is unsuitable, could be monitored closely with antibody titers in order to provide a timely treatment. In our case, the patient developed a bilateral optic neuritis with minimal visual impairment despite the administration of IvMP. It is tempting to speculate that the preventive therapy led to a milder clinical expression of the relapse.

Surprisingly, two patients had MOG antibodies below the cut-off during the acute phase with an increase during follow-up. This is hard to interpret, but could suggest that, at least in these patients, MOG antibodies are not the *primum movens* of the demyelination, but are produced as a response to myelin damage and antigen exposure caused by other mechanisms. Another possible explanation, as described for other anti-CNS antibodies, is that MOG itself could act as an "immunoprecipitator" of the antibodies that, being prevalently bound to their target, cannot be found in patient's serum or CSF.[82]

The clinical profile of our patients did not differ from other published cohorts.[66] We confirmed that the main clinical presentation is ADEM in children, and ON in adults.[70] As a novel finding, three patients showed radiological signs of PNS involvement. This has been described in patients with NMOSD and AQP4 antibodies,[83] but, to our knowledge, it has never been reported in MOGAD. PNS involvement with MOG antibodies is surprising, considering that MOG is considered a protein expressed exclusively in the CNS. Some studies, however, suggested that MOG can also be found in PNS myelin at lower concentration.[50] We previously described that, in TM, the presence of PNS involvement is a predictor of poor outcome.[84] Interestingly, in our cohort all the patients had an EDSS>3 during the acute phase, and 2/3 relapsed, but further studies are needed to confirm these findings.

In conclusion, we thoroughly analyzed antibody titers in a relatively large cohort of MOG positive patients, providing practical information on their interpretation and for

patient management. Our data support that titring MOG antibodies at disease onset and then every 3-6 months could help to stratify the relapse risk, and in some cases to predict relapse occurrence. In addition, we described for the first time a rare and unexpected subtype of MOGAD with PNS involvement. Larger studies are needed to confirm the prognostic value of such finding.

4 Acquired neuromyotonia in thymoma-associated myasthenia gravis: a clinical and serological study

4.1 Introduction

Acquired neuromyotonia (NMT) is characterized by spontaneous and continuous muscle fiber hyperactivity resulting from peripheral nerve hyperexcitability.[85,86] It associates with antibodies to CASPR2, LGI1, or both in around 40% of the cases, and shows good response to plasmapheresis.[20,87] These antibodies target neuronal cell-surface epitopes and thus are likely pathogenic.[88] NMT associates with myasthenia gravis (MG) in 15-21% of the patients,[89] but the peripheral nerve hyperexcitability symptoms such as muscle fasciculations and cramps might be overlooked because of the coexisting defect of neuromuscular transmission, or be misinterpreted as side effects of anti-acetylcholinesterase drugs.[90] Neurophysiologic assessments and testing for specific autoantibodies can aid the diagnosis.

Thymoma, an epithelial tumor typically associated with paraneoplastic neurological syndromes,[91] occurs in 25-30% of patients with MG,[92] and in up to 20% of those with NMT.[85] Its recurrence, which affects around 15% of thymectomized patients,[93,94] is insidious and has a poor prognosis, making early recognition important. Little is known about tumor prognosis in paraneoplastic NMT, and even less in the paraneoplastic NMT-MG comorbidity. Moreover, information on clinico-pathological variables and their relationships with neuronal cell-surface autoantibodies in patients with concurrent NMT, MG, and thymoma are also substantially lacking. In one case report the onset of NMT heralded thymoma recurrence in MG.[95] Recently, Torres-Vega et al identified antibodies to neuronal cell-surface antigens, the Netrin-1 receptors deleted in colon cancer (DCC) and uncoordinated-5A (UNC5A), in patients with MG, NMT, or both.[96]

To try to establish better the relationships between NMT, MG and thymoma, we studied patients with neuromyotonia extrapolated from a large series of thymoma-associated MG patients. We looked at the presence of autoantibodies to neuronal cell-surface antigens, and for predictors of thymoma recurrence.

4.2 Methods

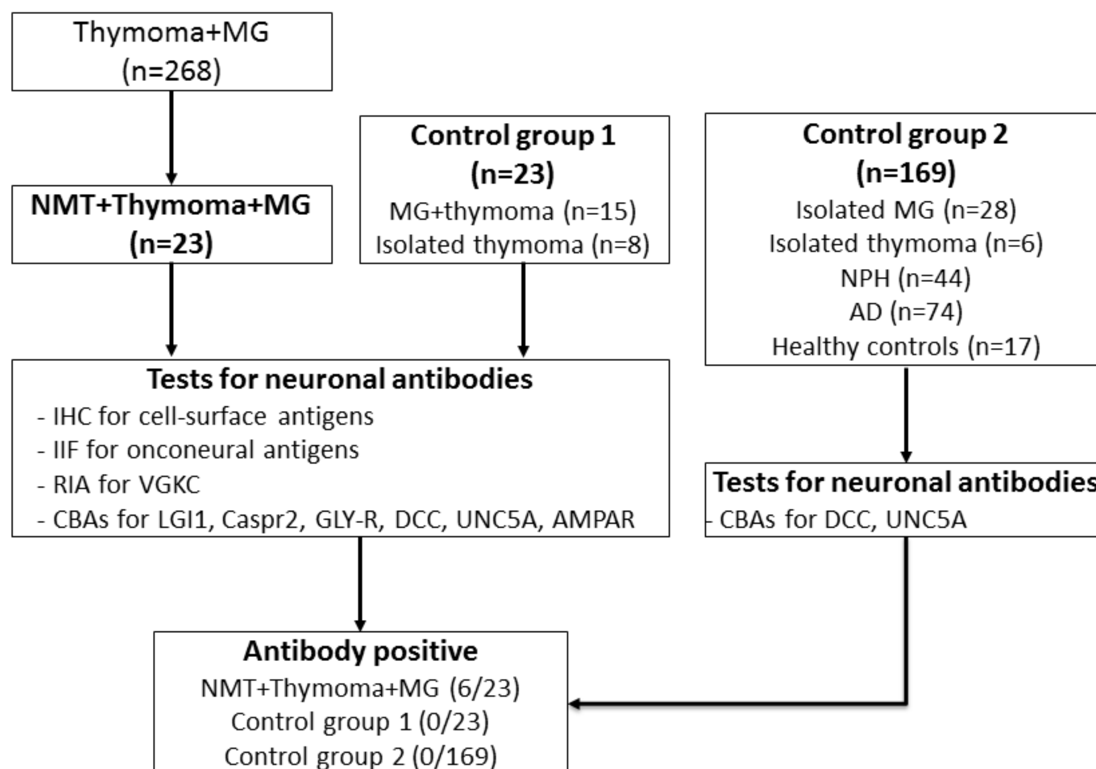
4.2.1.1 Subjects and materials

Participants included 23 patients comorbid for NMT selected from 268 consecutive MG patients with thymoma (all AChR-Ab positive) referred to the outpatient Clinics of Pisa (Italy) from 1990 to 2016 (Figure 24). Demographic and clinico-pathological information on all 268 patients were collected retrospectively. MG grading was assessed before thymectomy and at the end of follow-up according to the MG Foundation of America (MGFA) classification.[97] Thymoma staging was performed according to the World Health Organization (WHO)[98] and Masaoka system.[99] Patients were defined as having NMT if the following criteria were fulfilled: a) presence of cramps and/or muscle twitching (myokymia or fasciculations) affecting at least 2 skeletal regions; b) no pyridostigmine treatment or symptom persistence after drug suspension.[100]

Sera from the 23 NMT patients who fulfilled the criteria were collected close to onset of symptoms (median time from NMT onset, 8 months; IQR=5-33). Sera from thymoma-associated MG without NMT (n=15, from the original cohort) and MG without thymoma (n=8) were used as disease controls. To establish the specificity of assays for antibodies to the two Netrin-1 receptors, we used 152 sera from patients with thymoma with no neurological symptoms (n=6), MG without thymoma (n=28), and other neurological diseases (n=118; Alzheimer disease, 74; normal pressure hydrocephalus, 44), and also from 17 healthy individuals.

The study was approved by the Institutional Review Boards of the Institutes of Pisa and Pavia. Informed consent for antibody studies was obtained from all the patients.

Figure 24: workflow of the study



MG: myasthenia gravis; NMT: neuromyotonia; IHC: immunohistochemistry; IIF: indirect immunofluorescence; RIA: radio-immuno assay; VGKC: voltage-gated potassium channel; CBA: cell based assay; NPH: normal pressure hydrocephalus; AD: Alzheimer disease.

4.2.1.2 Antibody testing

Human embryonic kidney (HEK293T) cells were used for live cell-based assays (CBA) for antibodies to CASPR2, LGI1, AMPAR 1/2, Glycine receptor (GLYR) and UNC5A. A fixed CBA was used for DCC.[101] (see next section). As a screening technique and to confirm the CBA findings, immunohistochemistry on rat whole brain, modified in accordance with a protocol of tissue light fixation that preserves the native conformation of the neuronal antigens, was used.[102] Results were visualized on a fluorescence, or light microscope, and considered positive if the independent judgements of two blinded-to-

clinical-information investigators were concordant. For positive samples, endpoint titrations were obtained using serial dilutions.

VGKC complex antibodies were measured with a radioimmunoassay at the Oxford Neuroimmunology Laboratory[103] and AMPAR with a fixed CBA at the Barcelona Neuroimmunology Laboratory.[104] Sera were also tested for onconeural antibodies on primate cerebellum (indirect immunofluorescence kit, Euroimmun, Lübeck).

4.2.1.3 Cell-based assay

LCBA was performed as previously described for other antigens to test for LGI1, CASPR2, UNC5A and GlyR (see page 25-26). LGI1, CASPR and GLYR plasmids were kindly donated from Prof. Angela Vincent and Dr. Patrick Waters. UNC5A plasmid was purchased from OriGene (Carlsbad, California). The following dilutions were used: 1:40 dilution for LGI1, Gly-R and UNC5A; 1:100 dilution for Caspr2)

DCC antibodies were tested using a FCBA, that was performed as described at page 25-26. Cells were transfected using a reduced amount of DCC cDNA (1.5 ug/well; kindly provided by Prof. Masaki Fukata). After 10 hours from the transfection (longer transfection times led to increased background staining and non-specific binding of the secondary antibody on transfected cells), cells were removed from the incubator and used for staining. A commercial rabbit anti-human antibody against DCC (Protein tech, Manchester, UK) diluted 1:500 was used to assess the transfection.

Statistical analysis

Descriptive statistic was performed as described in other sections (page 45). To evaluate associations, univariate odds ratios (ORs) with 95% confidence interval (CI) were calculated. A logistic regression analysis was carried out to identify the mutually adjusted effect among endpoint (relapse in thymomatous-MG patients) and the independent variables. P-values $\leq$ 0.05 were considered significant (two-sided). All analyses were performed with STATA/SE for Windows, v14.

4.3 Results

4.3.1.1 Patients with thymomatous MG

Table 19 shows the clinico-demographic characteristics of the 268 patients. The median age at thymectomy was 49 years (interquartile range: 39-61), and M:F ratio was 1:1. 93.7% of patients had generalized MG, and 12.3% severe muscular weakness in bulbar, spinal, or both districts. MG preceded the detection of a thymoma in 92.5% of the cases. 94.6% of the patients had undergone thymectomy by the trans-sternal approach and 5.4% by the mini-invasive approach; the most frequent thymoma histology, B2,[98] was found in 26.9% of the cases. No patient had a thymic carcinoma. 152/268 patients (56.7%) received additional chemotherapy and/or radiotherapy. No data on residual thymic tissue were available.

Table 19: Clinico-demographic data in thymomatous MG patients

Patients, n (%)	All patients, 268 (100)	Monophasic, 236 (87.7)	Relapsing, 33 (12.3)	p-values
Months of follow-up, median (IQR)	41.5 (8-88)	33 (7-77)	87 (58-146)	<0.001
Age at thymectomy, median (IQR)	49 (39-61)	51 (41-62)	40 (31-47)	<0.001
Males, n (%)	134 (50)	121 (51.5)	13 (39.4)	0.193
Thymoma histology (WHO; ≥ B2), n (%)	171/264^ (64.8)	145/231^ (62.8)	26 (78.8)	0.072
Thymoma grading (Masaoka; ≥ IIb), n (%)	156/252^ (61.9)	127/221^ (57.5)	29/31 (93.6)	<0.001
Age at MG onset, median (IQR)	49 (38-61)	50 (40-62)	38 (29-45)	<0.001
MG grading before thymectomy (MGFA ≥ III), n (%)	84 (31.3)	73 (31.1)	11 (31.3)	0.792
MG onset before thymectomy, n (%)	248 (92.5)	220 (93.6)	28 (84.9)	0.073
MG duration (months) before thymectomy, median (IQR)	5 (2-13)	5 (2-12)	5 (2-26)	0.487
NMT, n (%)	23 (8.6)	15 (6.4)	8 (24.2)	0.003°

IQR, interquartile range; WHO, world health organization score; MG, myasthenia gravis; MGFA, MG foundation of America classification; NMT, neuromyotonia; ^, number on available patients

4.3.1.2 Patients comorbid for NMT and thymomatous MG

A combination of muscle twitching (including fasciculations, myokymia, or both) and cramps manifested in 23 patients (Table 20) suggestive of NMT. NMT onset was concomitant with MG onset in 4, or followed the diagnosis of MG with a median latency of 45 months (IQR, 12-81). Lower limbs were most commonly involved (17 patients), followed by the cranial region (periocular myokimias and/or facial fasciculations in 13 patients). EMG was performed in 12/23 patients, but neuromyotonic discharges were observed in only 3/12. Eight patients also had concomitant CNS involvement: a combination of sleep disorders, memory impairment and/or behavioral abnormalities (diagnosed as Morvan syndrome, n=3), isolated sleep disorders (n=2), seizures (n=1) and short-term memory loss (n=1); one patient had limbic encephalitis with a combination of seizures and short-term memory loss. Ten patients suffered from dysautonomia including hyperhidrosis (n=9), and severe gastrointestinal dysmotility with diarrhea (n=3). Overall, NMT symptoms improved in 12/14 patients treated with antiepileptic drugs alone (n=9), or in combination with immunosuppressant (n=3). In two, NMT symptoms were disabling and persistent despite treatments, one of whom died from thymoma recurrence.

Table 20: characteristics of thymomatous MG patients with NMT

Patients, n (%)	All patients, 23 (100)	Ab negative, 17 (73.9)	Ab positive, 6 (26.1)	p-value
Months of follow-up (from NMT onset), median (IQR)	45 (21-56)	44 (21-51)	49 (23-69)	0.599$^{\#}$
Months of follow-up (from thymectomy), median (IQR)	86 (62-121)	78 (62-95)	119.5 (98-151)	0.141$^{\#}$
Males, n (%)	8 (34.8)	4 (23.53)	4 (66.7)	0.131°
Age at MG onset, median (IQR)	49 (38-60.5)	42 (33-55)	35 (33-45)	0.219$^{\#}$
MGFA ≥ III before thymectomy, n (%)	7 (30.4)	6 (35.3)	1 (16.7)	0.621°
Osserman ≥ IIb before thymectomy, n (%)	15(65.2)	12 (70.6)	3 (50.0)	0.621°
Latency from MG to NMT, months, median (IQR)	45 (12-81)	44 (12-73)	76.5 (35-107)	0.161$^{\#}$
Age at NMT presentation, mean (SD)	46 (36-57)	46 (39-56)	41.5 (33-57)	0.420$^{\#}$
NMT symptoms				
cramps, n (%)	20 (87)	15 (88.2)	5 (83.3)	>0.90
myokymias, n (%)	17(73.9)	14(82.4)	3(50.0)	0.279°
fasciculations, n (%)	10(43.5)	5(29.4)	5(83.3)	0.052°
muscle twitching n (%)	20(87.0)	14(82.4)	6(100.0)	0.539°
NMT distribution				
cranial n (%)	13 (56.5)	9 (52.9)	4 (66.7)	0.660°
trunk n (%)	1 (4.4)	0 (0.0)	1 (16.7)	0.261°
upper limbs n (%)	11 (47.8)	8 (47.1)	3 (50.0)	>0.90°
lower limbs n (%)	17 (73.9)	13 (76.5)	4 (66.7)	0.632°
Additional Symptoms				
tremors n (%)	15 (62.5)	11 (64.7)	4 (66.7)	>0.90°
muscular rigidity n (%)	10 (43.5)	7 (41.2)	3 (50.0)	>0.90°
paresthesia n (%)	7 (30.4)	4 (23.5)	3 (50.0)	0.318°
pain n (%)	8 (34.8)	4 (23.5)	4 (66.7)	0.131°
weight loss n (%)	4 (17.4)	1 (5.9)	3 (50)	0.004°
CNS involvement, n (%)	8 (34.8)	3 (17.6)	5 (83.3)	0.009°
confusion/memory impairment, n (%)	3 (13.0)	2 (11.8)	1 (16.7)	>0.90°
sleep disorders, n (%)	5 (21.7)	2 (11.8)	3 (50.0)	0.089°
seizures, n (%)	2 (8.7)	0 (0)	2 (33.3)	0.059°
Dysautonomia, n (%)	10 (43.5)	5 (29.4)	5 (83.3)	0.052°
hyperhidrosis, n (%)	9 (39.1)	4 (23.5)	5 (83.3)	0.018°
arrhythmias, n (%)	4 (17.4)	2 (11.8)	2 (33.3)	0.270°
gastrointestinal dysmotility, n (%)	3 (13.0)	1 (5.9)	2 (33.3)	0.155°
Thymoma WHO (≥ 2B), n (%)	12 (52.2)	8 (47.1)	4 (66.7)	0.640°
Thymoma recurrence, n (%)	8 (34.8)	4 (23.5)	4 (66.7)	0.050°
NMT response to therapy, n (%)	12/14 (85.7)	8 (47.1)	4 (66.7)	0.126°
MG Outcome n (%)				
improved	5 (21.7)	4 (23.5)	1 (16.7)	
minimal manifestations	8 (34.8)	6 (35.3)	2 (33.3)	
pharmacological remission	7 (30.4)	5 (29.4)	2 (33.3)	0.627°
complete stable remission	2 (8.7)	2 (11.76)	0 (0.0)	
death	1 (4.3)	0 (0.0)	1 (16.7)	

Ab, antibody; NMT, neuromyotonia; IQR, interquartile range; MG, myasthenia gravis; MGFA, MG Foundation of America classification; WHO, World Health Organization score; SD, standard deviation; °Fisher's Exact Test; # Mann-Whitney Test

Autoantibody reactivities

Six of the 23 NMT patients had autoantibodies against neuronal cell-surface proteins. Table 21 shows their clinico-demographic characteristics. In 5/6 patients the thymoma histology was B2. CASPR2 and/or LGI1 antibodies were found in 4 patients, one patient had antibodies to the Netrin-1 receptor DCC, and the patient with limbic encephalitis was positive for AMPAR antibody. Two patients showed polyreactivities that included the two Netrin-1 receptors (CASPR2+LGI1+DCC+UNC5A and CASPR2+LGI1+DCC, see Figure 2). Both patients had features consistent with the diagnosis of Morvan syndrome, and both had a thymoma recurrence. Fasciculations (p=0.017) and CNS involvement (p=0.04) were more frequent in autoantibody-positive NMT patients than in those without these antibodies.

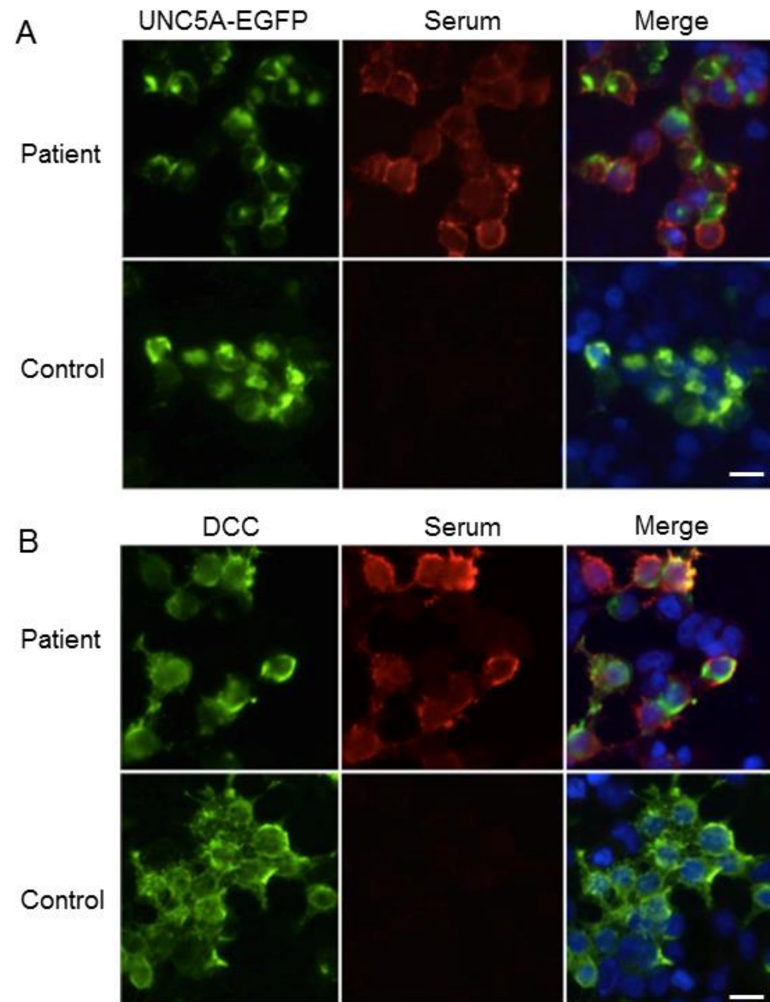
No seroreactivity was found in patients with MG without thymoma or thymoma-associated MG without NMT, and all control sera were negative for DCC and UNC5A antibodies (Figure 25). The 4 samples positive for LGI1 and/or Caspr2 antibodies were confirmed on immunohistochemistry, which showed a neuropilar pattern consistent with that of each antibody. Only 1 additional patient showed a hippocampal neuropilar staining, that was characterized as AMPAR antibodies in Barcelona Neuroimmunology Laboratory (Prof. Francesc Graus). The patient with isolated DCC antibodies showed no specific staining on immunohistochemistry. The 4 LGI and/or Caspr2 samples had VGKC above or right below the 100 pM cut-off (Figure 26). All the samples were negative for onconeural antibodies and for GLYR antibodies.

Table 21: Characteristics of thymomatous myasthenia gravis patients with neuromyotonia and antibodies against neuronal cell-surface antigens

N	Sex	Age	MG stage	Thymoma (WHO)	Additional treatment	Timing of recurrence after thymectomy	NMT onset	Main NMT symptoms	Accessory symptoms	CNS symptoms	Antibodies (Endpoint titre)	NMT Treatment	Response to treatment	EMG neuromyotonic discharges	Final Diagnosis	MG Outcome
1	F	47	IIA	B2	Chemo/RT	3 and 7 y	6 months before second relapse	Orbicular and abdominal myokimias	Hyperhidrosis	None	Caspr2 (200)	LEV	Good	No	NMT	Minimal Manifestation
2	M	29	IIB	B2	RT	6 and 9 y	Concomitant with first relapse	Severe ocular myokymias, cramps and fasciculations in upper and lower limbs	Paresthesias in lower limbs	Seizures, memory impairment	AMPAR (400)	CBZ	Good	Yes	NMT+ limbic encephalitis	Minimal Manifestation
3	F	33	IVB	B2	No	No recurrence	1 month after thymectomy	Ocular myokymias, fasciculations in upper limbs	Paresthesias in lower limbs, hyperhidrosis, arrythmia	Sleep disorder	LGI1 (160)	PNT	Good	No	NMT	Improved
4	M	57	IIB	B2	No	No recurrence	5 years after thymectomy	Severe ocular myokymia; diffuse cramps and fasciculations	Hyperhidrosis	None	DCC (12000)	PNT	Good	No	NMT	Pharmacological remission
5	M	36	IIA	B1	Chemo	3 y	3 months before relapse	Fasciculations and cramps in lower limbs	Burning pain in lower limbs, hyperhidrosis	Sleep disorder, personality change	Caspr2 (800)+LGI1 (640)+ DCC (900)+UNC5A (2700)	PNT, PRG, CBZ	Good	No	Morvan syndrome	Pharmacological remission
6	M	57	I	B2	Chemo	9 y	After relapse	Cramps and fasciculation in lower limbs	Paresthesias in lower limbs	Seizures, sleep disorder, memory impairment	Caspr2 (200)+LGI1 (320)+ DCC (300)	None	n.a.	Yes	Morvan syndrome	Death (from neoplastic disease)

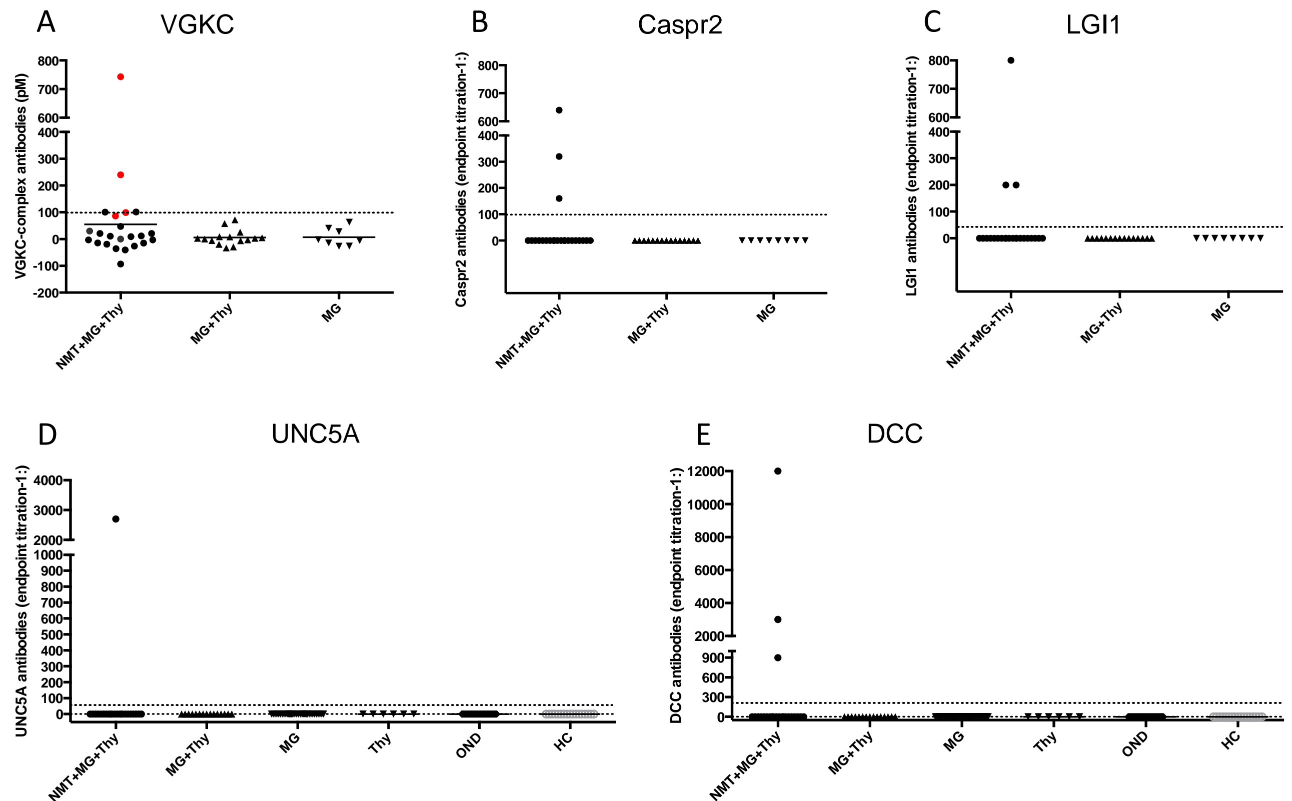
MG, myasthenia gravis; WHO, World Health Organization score; Chemo, chemotherapy; NMT, neuromyotonia; CNS, central nervous system; EMG, electromyography; RT, radiotherapy; DCC, deleted in colon cancer; UNC5A, uncoordinated-5A; PNT, phenoytoin; LEV, levetiracetam; CBZ, carbamazepine; PRG, pregabalin; n.a., not applicable

Figure 25: cell-based assays for Netrin-1 receptor antibody detection



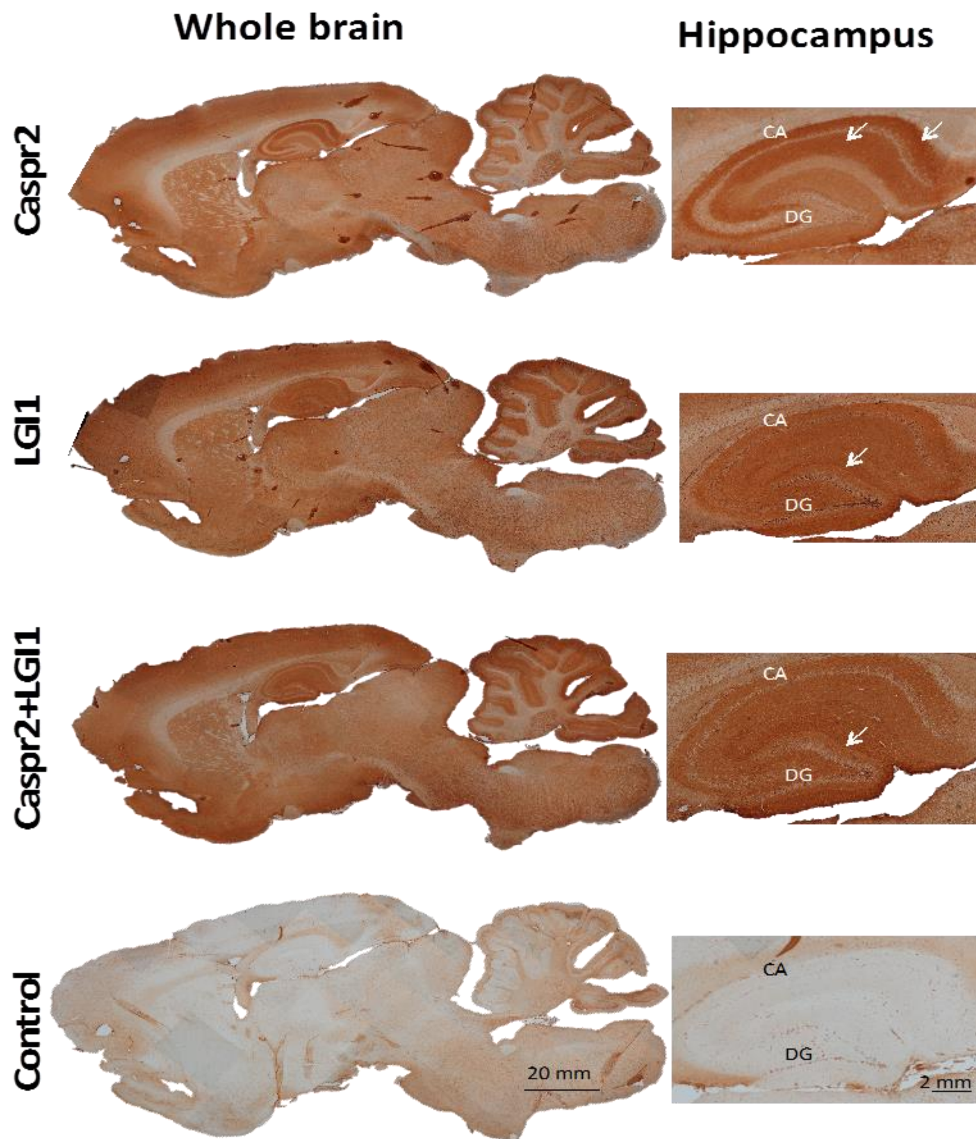
HEK293T cells transfected with either EGFP-tagged UNC5A (A), or commercial fluorescent antibody against DCC (B) (green, left panels). IgG from patients, but not from controls, bind the cells (red, central panels), co-localizing with the EGFP-tagged UNC5A, or with the DCC antibody (merging, right panels; DAPI stains nuclei). Scale bar, 20 µm.

Figure 26: antibody titers in patients and controls



Antibodies to neuronal cell-surface antigens were found in patients with neuromyotonia (NMT)+myasthenia gravis (MG)+thymoma, but not in controls with MG+thymoma, or isolated MG (A,B,C). Red dots in panel A represent patients positive for LGI1 and/or Caspr2 antibodies. UNC5A and DCC were not found in any additional controls (D,E). Thy, thymoma; OND, other neurological diseases; HC, healthy controls

Figure 27: Staining patterns of LGI1, CASPR2, and LGI1 plus Caspr2 antibodies on rat brain tissue



Examples of the staining patterns provided by sera (1:200 dilution) of thymomatous MG patients with NMT positive for Caspr2, LGI1 or both antibodies. Caspr2 provides a hippocampal staining more intensely localised in the Cornus Ammonis (CA) neuropilum (white arrows) compared to the Dentate Gyrus (DG). LGI1 provides a typical staining in the DG that involves more intensely the outer third of the molecular layer (white arrow). When Caspr2 and LGI1 antibodies coexist, the LGI1 staining tends to prevail, making the identification of both antibodies just from the staining pattern very difficult (white arrow). No neuropilar staining is present with a control serum.

Predictors of thymoma recurrence

During follow-up, 33/268 (12%) patients developed a recurrence of the thymoma after a median time of 46.1 months (IQR, 26.5-63.5) and 18/33 had more than 1 recurrence. Table 22 reports the clinico-pathological factors associated with tumor recurrence. In both univariate and multivariate analysis younger age at thymectomy (OR, 0.94; 95%CI, 0.91-0.97), higher staging of the original thymoma (Masaoka stage ≥IIb, OR, 13.21; 95%CI, 2.88-60.48), and co-existence of NMT (OR, 7.45; CI, 1.97-28.17) predicted tumor recurrence. Serological results were not considered as they were a subset of NMT. No differences in tumor recurrence were found when comparing thoracotomy and mini-invasive surgery. Notably, the onset of neuromyotonic symptoms preceded the discovery of thymoma recurrence in 6/8 patients, and the latency between the diagnosis of NMT and thymoma recurrence ranged between 3 and 12 months.

Table 22: Univariate and multivariate analysis for predictors of tumour recurrence in thymomatous MG patients

	Univariate, OR (CI)	p-values	Multivariate, OR (CI)	p-values
Age at thymectomy, median	0.95 (0.93-0.97)	<0.001	0.94 (0.91-0.97)	<0.001
Male sex	0.61 (0.29-1.29)	0.194	-	-
Thymoma histology (WHO; ≥ B2)	2.20 (0.91-5.33)	0.072	-	-
Thymoma grading (Masaoka; ≥ IIb)	10.73 (2.38-48.36)	<0.001	13.21 (2.88-60.48)	0.001
Age at MG onset, median	0.94 (0.92-0.96)	<0.001	-	-
MG grading before thymectomy (MGFA ≥ III)	1.11 (0.51-2.41)	0.793	-	-
MG onset before thymectomy	0.38 (0.13-1.14)	0.073	-	-
MG duration (months) before thymectomy	1.01 (1.00-1.01)	<0.001	-	-
NMT	4.69 (1.76-12.46)	<0.001	7.45 (1.97-28.17)	0.003

OR, odds ratio; CI, confidence interval; WHO, world health organization score; MG, myasthenia gravis; MGFA, MG foundation of America classification; NMT, neuromyotonia; ^, number on available patients

4.4 Discussion and conclusions

This study focused on the clinical and serological characterization of patients with MG, NMT and thymoma recurrence, extrapolated from a very large series of patients with MG and thymoma, who were investigated for tumor-related prognostic factors. We identified younger age at thymectomy and the occurrence of NMT as novel factors predictive of thymoma recurrence. In addition, we confirmed that the recently identified autoantibodies to netrin-1 receptors can be found in thymoma-associated MG with NMT comorbidity.

In the literature, there is only one report on a patient with overlapping MG, NMT, and thymoma linked to the co-occurrence of NMT and thymoma recurrence.[95] Our data give statistical weight to this observation and strongly underpin the importance of proper tumor vigilance and screening in patients with MG receiving the diagnosis of NMT and *vice versa*. In the most common association with MG, but without NMT, thymoma prognosis has been variably reported as better or worse.[105,106]

The advanced Masaoka stage was a strong predictor of tumor recurrence, in line with the literature data,[94] whereas there was no significant association between thymoma histology according to WHO classification and tumor recurrence. This apparent discrepancy is likely related to the high representation, in our patients, of the most commonly MG-associated B2-B3 stage.[105] The evaluation of incomplete tumor resection as another predictor of thymoma recurrence was unavailable due to the lack of data on residual thymic tissues

Due to the retrospective nature of the study, NMT was exclusively identified on the basis of clinical criteria. Half of our patients underwent EMG, and a minority of them showed the NMT typical myotonic discharges. This is not surprising, as it is established that around half of the patients with NMT lack disease-specific EMG features.[100] As a further support for the diagnosis of NMT, symptoms improved with sodium channel-blocking drugs in almost all patients.

The subgroup of patients with thymoma-associated MG and NMT showed reactivities to known (CASPR2 and LGI1), recently identified (Netrin-1 receptors), and unexpected (AMPAR) neuronal cell-surface antibodies. Overall, the presence of neuronal cell-surface autoantibodies was more frequent in patients with recurrent thymoma but only 26.1 % in NMT overall. In addition, in four of the six seropositive patients, the onset of NMT was very close to the recurrence of the thymomas. Only

two studies reported the presence of Netrin-1 receptors autoantibodies, more frequently to DCC than UNC5A and almost invariably coexisting with CASPR2 antibodies, in patients with thymoma-associated Morvan syndrome,[101] or comorbid for thymoma-associated MG and NMT.[96] In partial contrast, we found the coexistence of Netrin-1 receptors antibodies (DCC plus UNC5A, or DCC alone) with both CASPR2 and LGI1 antibodies in two patients with Morvan syndrome and thymoma recurrence. Taking into account the Netrin-1 receptor involvement in the CNS synaptic plasticity[107] and in axonal growth and guidance at the paranodes of Schwann cell axons,[108] a possible pathogenic role for these autoantibodies is possible, but purely speculative at present. However, in the cases of multiple polyspecific autoantibody reactivities, determining which immune response is pathogenic and which epiphenomenal is often difficult to ascertain.

From a clinical point of view, Netrin-1 receptor antibodies seem to be highly specific of paraneoplastic NMT or Morvan syndrome, as they were absent in patients with MG without thymoma, thymoma-associated MG without NMT, thymoma with no neurological symptoms, or other neurological diseases. Rare associations with isolated MG or thymoma have been reported.[96,101] In accordance with other studies,[38,96,101] also in our series CASPR2 antibodies were the most frequent autoantibody reactivity associated with thymoma, and, as a whole, the autoimmunity to neuronal cell-surface antigens developed at advanced stages of the tumor. Taken together, these observations stress the prognostic importance of testing for CASPR2 and LGI1 antibodies in patients with thymoma-associated MG and NMT, and suggests that, to reinforce the prognostic goals, centralized laboratory services could guarantee the availability of testing for other rarer or unexpected autoantibody reactivities to neuronal cell-surface antigens. Our results, indeed, expand the findings by Torres-Vega and colleagues, confirming the utility of testing for such tumor-associated autoantibodies as useful biomarkers of thymoma in patients with autoimmune neuromuscular or CNS diseases.[96] The confirmation includes the entry of Netrin-1 receptor antibodies into the realms of the cancer-predicting antibodies that identify patients requiring stringent and prolonged tumor surveillance. Our data also confirm that testing for VGKC antibodies has little if no clinical relevance.[109,110]

In conclusion, our study suggests that NMT is a potentially underdiagnosed condition in thymomatous MG patients, , and can be helpful to predict and timely manage thymoma recurrence, which is usually insidious, tending for late local

occurrence even after complete thymectomy.. It also defined the frequency and diagnostic utility of antibodies to neuronal cell-surface antigens associated with thymoma in patients comorbid for NMT and MG. This information provide a useful tool for patients management and appropriate tumour risk stratification in such clinical setting

5 Summary of conclusions

The main products of this thesis concern the implementation of advanced autoantibody-detection techniques at the Pavia Neuroimmunology Laboratory, and, as a remarkable novelty, the investigation of their laboratory impact on clinical variables in large series of patients .

In autoimmune encephalitis, we showed that advanced techniques based on murine tissue screening and in-house cell based assays performed better than the commercially available kits, In addition, these techniques allowed us to move the first steps toward the characterization of novel autoantibody reactivities, through precipitation experiments, and to identify a range of potential candidate proteins as antigenic targets in autoimmune encephalitis. From a clinical point of view, we studied a large retrospective cohort of autoimmune encephalitis selected according to Graus et al., diagnostic criteria.[43] We characterized for the first time a subgroup of patients with seronegative autoimmune encephalitis, and established that the limbic phenotype is the most commonly identified. We showed that seropositive and seronegative autoimmune encephalitis share common features, such as relapse rate and tumor frequency. We also showed that, as a whole, seronegative autoimmune encephalitis has a worse prognosis compared to seropositive, possibly due to differences in treatment strategies, which are more prompt and aggressive when an autoantibody has been detected. Again, in this cohort we confirmed the importance of in-house antibody testing, that allowed the correct clinical classification of about 1/3 of patients negative to the commercial first line screening tests.

In demyelinating disorders, we first dwelled into comparing different in-house and commercial tests for MOG antibodies detection, and showed that a combinatorial multistep approach led to the highest diagnostic accuracy. Then, we used this multistep approach to identify a large cohort of adult and pediatric patients with MOG antibodies, and characterize them from a clinical and serological point of view over long follow-ups. We identified for the first time 3 patients with radiological features of PNS involvement in MOG associated disorders, providing a thorough description of the cases. The longitudinal studies allowed us to associate higher titers at onset with a relapsing course. In addition, we showed that individual titers kinetics can predict the onset of new relapses.

Finally, we explored a niche topic, investigating prognostic factors in patients with thymoma and myasthenia gravis. We identified the occurrence of acquired neuromyotonia as a predictor of thymoma relapse. In addition, along with known neuronal antibodies such as CASPR2 and LGI1, we implemented two new CBAs for the netrin-1 receptor antibodies, a novel antigen identified in patients with neuromyotonia, finding them in a subgroup of patients with thymoma, myasthenia and neuromyotonia with higher risk of thymoma recurrence.

6 Future directions

In the past years, the implementation of efficient antibody screening techniques has led to the identification of an increasing number of neuroglial antibodies. Interestingly, a similar laboratory paradigm consisting of screening on murine tissue and characterization of the staining patter has been used to identify autoantibodies in very different clinical setting, such as AQP4 antibodies in NMO and NMDAR antibodies in AE. In recent times, however, the number of new antigens characterized has finally reached a plateau, and the epidemiological weight of the latest antigens characterized, such as Neurexin-3α is very limited.[111] In the field of demyelinating disorders, screening systems are even less efficient, and apart from AQP4 and MOG no useful novel antibodies have been identified in the recent years. Despite experimental evidence supporting the presence of demyelinating autoantibodies in MS, no reliable antigenic target has been characterized so far.[112,113] One on of the possible reasons for this failure could be found in the relatively low degree of homology between the human and murine antigenic repertoire. As for pathogenic MOG antibodies, that in most cases recognize only the human isoform of the protein, it is possible that our current screening methods largely relying on murine tissue miss relevant autoantibodies directed exclusively against human antigens. Novel screening substrates are necessary in order to expand our current list of autoantibodies, possibly built on systems able to reflect the human antigenic repertoire. Possibilities for the future include, among others, cell lines derived from induced pluripotent stem cells with neuroglial differentiation.[114]

On the pathogenic side, there are still several open questions on the mechanisms that lead to antibody penetration into the brain parenchyma. Whilst many studies investigated the mechanisms that lead to activated T-cells migration across the blood-brain barrier and the choroid plexus,[115] few data are available on how peripherally produced autoantibodies can reach their targets within the CNS. Recent studies suggest that, in AQP4 antibody-associated diseases, additional autoantibodies directly targeting the blood-brain barrier could be responsible for the increased tight junction permeability that, in turn, could facilitate the entry of AQP4 targeting antibodies into the CNS.[116] Future studies will need to elucidate the role of blood-

brain barrier in autoantibody mediated disorders, and the mechanisms that can allow pathogenic antibodies to cross the barrier.

Finally, the cellular mechanisms that lead to the development and persistence of autoreactive B-clones targeting brain antigens are just starting to be clarified. Recent studies highlighted that preformed B autoreactive cells possess anti-AQP4 reactivity, and that that specific cytokine signatures are implicated in antibody secretion.[117] However, this results were obtained with whole lymphocyte cultures from affected patients, that prevented the identification of antibody producing cells. Future studies, exploiting high-throughput systems to investigate the memory B-cell repertoire, could help provide a molecular characterization and insight on pathogenic mechanisms of the antibody producing cells.[118] Advances in these fields will be essential to find novel and tailored therapeutic strategies in the autoantibody-mediated disorders of the nervous system.

7 List of abbreviations

AChR: acetylcholine receptor

ADEM: acute disseminated encephalomyelitis

AE: autoimmune encephalitis

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ANOVA: analysis of varance

CNS: central nervous system

CSF: cerebrospinal fluid

S-Gabs: glial surface autoantibodies

CASPR2: contactin Associated Protein 2

CBA: cell based assay

CCBA: commercial cell based assay

CI: confidence interval

DAB: diaminobenzidine

DAPI: 4′,6-diamidino-2-phenylindole

DMEM: Dulbecco's eagle modified essential medium

DNA: desoxyribonucleic acid

DPPX: dipeptidyl aminopeptidase-like protein 6

EAE: experimental autoimmune encephalitis

EGFP: emerald fluorescent green protein

ELISA: enzyme-linked immunosorbent assay

FBDS: faciobrachial dystonic seizures

FCBA: fixed cell based assay

$GABA_A R$: γ-aminobutyric acid A receptor

GABABR: γ-aminobutyric acid B receptor

GAD: glutamic acid decarboxylase

GLYR: glycine receptor

HBSS: Hank's balanced salt solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEK293: human embryonic kidney-derived 293 cells

IC-NAbs: intracellular neuronal antibodies

IgLON5: IgLON Family Member 5

IHC: immunohistochemistry on rat brain (optimized for surface antigens)

IQR; interquartile range

LCBA: live cell based assay

LE: limbic encephalitis

LGI1: leucine-rich glioma inactivated protein 1

MG: myasthenia gravis

mGluR5: metabotropic glutamate receptor 5

MOGAD: MOG antibodies associated disorders

MuSk: muscle-specific kinase

NEG-AE: seronegative autoimmune encephalitis

NET: neuroendocrine tumor

NF-155: neurofascin 155

NMDAR: N-methyl-D-aspartate receptor

NMT: neuromyotonia

NORSE: new onset refractory status epilepticus.

ON: optic neuritis

OND: other neurological disorders

PBS: phosphate buffer saline

PFA: paraformaldehyde

POS-AE: seropositive autoimmune encephalitis

POSS-AE: possible autoimmune encephalitis

SDS-PAGE: sulphate polyacrylamide gel electrophoresis

S-Nabs: neuronal surface anutoantibodies

S-NGAbs: neuroglial surface autoantibodies

PNS: peripheral nervous system

SIADH: syndrome of inappropriate antidiuretic hormone secretion

TM: transverse myelitis

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