Autoimmune neurological syndromes with anti-CASPR2 antibodies : clinical, immunological and genetic characterization

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RÉSUMÉ

Les auto-anticorps dirigés contre CASPR2 (Contactin-2 Associated Protein), une protéine d'adhésion neuro-gliale, ont été décrits dans au moins trois syndromes neurologiques auto-immuns: l'encéphalite limbique auto-immune, la neuromyotonie acquise (aussi appelée syndrome d'Isaacs) et le syndrome de Morvan. Cependant, le phénotype clinique associé aux anticorps anti-CASPR2 demeure imparfaitement décrit. En particulier, les spécificités cliniques de l'encéphalite limbique associée aux anticorps anti-CASPR2 n'ont pas été décrites de façon détaillée lorsque ces anticorps ont été rapportés pour la première fois. En outre, la définition du syndrome de Morvan est imprécise et repose sur des descriptions historiques remontant au milieu du vingtième siècle. De plus, certains auteurs considèrent que les patients porteurs d'anticorps anti-CASPR2 ne forment pas de syndromes spécifiques, mais présentent des associations aléatoires d'une série de symptômes clés. Enfin, les facteurs physiopathologiques qui sous-tendent les variations du phénotype neurologique des patients avec anticorps anti-CASPR2, sont inconnus.

Au cours de ce travail de thèse, nous abordons le problème de la caractérisation clinique des patients avec anticorps anti-CASPR2. Nous utilisons une cohorte rétrospective nationale de patients chez lesquels ont été détectés des anticorps anti-CASPR2, afin de décrire la présentation clinique de l'encéphalite limbique auto-immune associée à ces anticorps, et du syndrome de Morvan, de préciser le pronostic de l'encéphalite limbique à anticorps anti-CASPR2, et d'analyser la répartition des symptômes des patients afin de vérifier si ceux-ci se distribuent de manière aléatoire ou forment bien des profils cliniques particuliers. Ce travail de thèse est divisé en trois études.

La première étude est consacrée à l'analyse de la présentation clinique et du pronostic de l'encéphalite limbique auto-immune à anticorps anti-CASPR2. Nous observons que la majorité des patients sont des hommes âgés de 50 à 75 ans, et ont fréquemment des symptômes extra-limbiques, en particulier une ataxie cérébelleuse. Ces patients répondent le plus souvent aux immunothérapies, bien qu'un quart des patients garde des séquelles cognitives, une épilepsie, ou une ataxie. Dans la seconde étude, nous décrivons pour la première fois l'ataxie épisodique auto-immune, un symptôme jusqu'ici exclusivement associé à l'encéphalite auto-immune avec anticorps anti-CASPR2. Ce symptôme est similaire aux ataxies épisodiques héréditaires et, de façon remarquable, nous avons décelé chez 2 patients un polymorphisme rare des gènes KCNA1 ou CACNA1A, qui sont impliqués respectivement dans l'ataxie épisodique de type 1 et de type 2. Bien que l'impact de ces variants rare sur la fonction des canaux ioniques qu'ils codent est inconnu, ces observations soulèvent la question de l'influence du terrain génétique sur la détermination du phénotype neurologique des patients avec anticorps anti-CASPR2. Dans la troisième étude, nous réalisons une analyse typologique des symptômes des patients dans le but de vérifier si les symptômes se distribuent de façon aléatoire ou au contraire forment des profils cliniques spécifiques. Par cette méthode, nous démontrons que les symptômes s'associent de façon non-aléatoire, permettant de classer les patients en trois groupes distincts, qui correspondent à l'encéphalite limbique auto-immune, à la neuromyotonie, et au syndrome de Morvan. De plus nous confirmons la grande variété de présentation clinique de l'encéphalite à anticorps anti-CASPR2, qui ne se limite pas à la présence de symptômes limbiques, puisque plus d'un tiers de patients présente également des symptômes extra-limbiques, tels que l'ataxie cérébelleuse, la dysautonomie, la perte de poids, ou des mouvements anormaux. De façon notable, moins de dix pour cent des patients avaient une combinaison de symptômes limbiques et de neuromyotonie. Enfin, le syndrome de Morvan se caractérise dans notre série par des signes sévères d'hyperexcitabilité nerveuse

périphérique, des signes sévères de dysautonomie, une insomnie sévère, une perte poids fréquente, et une association au thymome malin. Cette classification clinique en trois syndromes est confortée par des variations des caractéristiques des auto-anticorps, puisque les patients avec une encéphalite limbique ont des titres sériques d'anticorps anti-CASPR2 plus élevés, et des anticorps anti-CASPR2 détectable plus souvent dans le liquide céphalo-rachidien, ainsi que par des différences en terme d'haplotype HLA associé, puisque les patients avec un syndrome de Morvan ne sont pas porteurs de l'haplotype HLA DRB1*1101 qui est retrouvé chez 94% des patients avec une encéphalite limbique à anticorps anti-CASPR2.

En conclusion, ce travail de thèse conforte la notion que les patients avec anticorps anti-CASPR2 présentent des syndromes spécifiques, l'encéphalite limbique, la neuromyotonie acquise, et le syndrome de Morvan, avec une association de deux de ces syndromes chez seulement une minorité de patients. Ce travail permet de décrire en détail les présentations cliniques de l'encéphalite limbique à anticorps anti-CASPR2 et du syndrome de Morvan. Les titres et site de détection des anticorps anti-CASPR2, l'association aux thymomes, et les associations HLA vont dans le sens de cette classification et suggèrent que la variabilité clinique observée avec les anticorps anti-CASPR2 est liée à l'existence de mécanismes physiopathologiques différents selon les syndromes.

ABSTRACT

Antibodies against CASPR2 (Contactin-2 Associated Protein), a neuroglial cell-adhesion protein, have been described in at least three neurological syndromes: autoimmune limbic encephalitis, acquired neuromyotonia (or Isaacs' syndrome) and Morvan syndrome. However, the clinical phenotype associated with anti-CASPR2 antibodies is not yet completely understood. First, the clinical specificities of autoimmune encephalitis with anti-CASPR2 antibodies were not detailed in the first reports of anti-CASPR2 antibodies patients. Moreover, the definition of Morvan syndrome is vague and relies on historical descriptions dating back from the middle of the twentieth century. In addition, some authors consider that instead of specific syndromes, anti-CASPR2 antibodies associate with a set of core symptoms that combine randomly in the patients. Last, the pathophysiologic factors underpinning clinical variability in the anti-CASPR2 antibodies patients are unknown.

In this PhD project, we use a nationwide, retrospective cohort of anti-CASPR2 antibodies patients, in order to address the issue of the clinical characterization of anti-CASPR2 antibodies patients. We aimed at describing the clinical presentation of CASPR2 encephalitis and Morvan syndrome, studying the outcomes of CASPR2 encephalitis, and analyzing the repartition of the patients' symptoms in order to assess if the symptoms are distributed randomly or if instead they form distinct clinical patterns. The present PhD project is divided into three studies.

In the first study, we analyze clinical presentations and outcomes of anti-CASPR2 antibodies patients with limbic encephalitis. We observe that most patients were males from 50 to 75 years old, and frequently had extra-limbic symptoms, such as cerebellar ataxia. In addition, response to immunotherapy was good, even though 25% of the patients did not return to baseline and were left with residual symptoms, including cognitive disturbances, epilepsy, and cerebellar ataxia. In the second study, we describe the first reported cases of

autoimmune episodic ataxia, a novel symptom that so far has been found only in anti-CASPR2 antibody associated autoimmune limbic encephalitis patients. It consists in transient episodes of paroxysmal ataxia, and is reminiscent of hereditary episodic ataxia. Interestingly, we found in two patients rare variants of CACNA1A and KCNA1, two genes involved in the main types of hereditary episodic ataxia. While the impact of these variants on ion channel functions is unknown, it raises the question of the role of the genetic background in phenotype determination in anti-CASPR2 antibodies patients. In the third study, we use a statistical cluster analysis to assess anti-CASPR2 antibodies patients' symptoms combinations. We found that the symptoms do not form random combinations, but that instead clinical patterns can be identified, which correspond to patients with limbic encephalitis, Morvan syndrome, and neuromyotonia. In addition, we confirm the expansive clinical presentation of limbic encephalitis, since more than a third of the patients had non-limbic symptoms such as cerebellar ataxia, dysautonomia, weight loss, and movement disorders. Notably, less than ten percent of the patients had a combination of neuromyotonia and limbic symptoms. Finally, the Morvan syndrome patients had severe peripheral nerve hyperexcitability features, severe dysautonomia, severe insomnia, weight loss, and frequently had a malignant thymoma. This clinical classification into three specific syndromes is supported by differences in term of autoantibody specificities, as limbic encephalitis patients tended to have higher anti-CASPR2 antibodies levels and were more frequently cerebrospinal fluid-positive, and by the genetic background, since the Morvan syndrome patients did not have the HLA DRB1*1101 association that is found in limbic encephalitis patients.

In conclusion, the present PhD project supports the view that anti-CASPR2 antibodies patients can be classified into three specific syndromes, autoimmune limbic encephalitis, neuromyotonia, and Morvan syndrome, which are found associated in only a minority of the patients. In addition, the present PhD project provides detailed descriptions of the clinical

presentations of anti-CASPR2 antibody-associated autoimmune limbic encephalitis and Morvan syndrome. Importantly, autoantibody specificities, thymoma association, and HLA allele associations support the clinical classification and suggest that differences in etiopathogeny account for the clinical variability observed in anti-CASPR2 antibodies patients.

CASPR2 ANTIBODIES: CURRENT CONCEPTS, PENDING ISSUES

INTRODUCTION

Antibodies against CASPR2 (Contactin-2 Associated Protein), a neuroglial cell-adhesion protein, have been described in at least three neurological syndromes: autoimmune limbic encephalitis, acquired neuromyotonia (or Isaacs' syndrome) and Morvan syndrome (Irani et al. 2010; Lancaster et al. 2011; Irani et al. 2012). Since these syndromes encompass symptoms of the central nervous system (e.g. amnesia, seizures, cerebellar ataxia, sleep disorders, or hyperkinetic movement disorders) as well as of the peripheral nervous system (e.g. myokimia, fasciculations, and neuropathic pain), the clinical spectrum associated with anti-CASPR2 antibodies (CASPR2-Abs) is wide. Several clinical and historical setbacks have made difficult the establishment of a robust clinical classification of CASPR2-Abs patients. First of all, anti-CASPR2 antibodies have been described relatively recently, and before that the antibodies found in patients with acquired neuromyotonia, Morvan syndrome, or autoimmune limbic encephalitis, were thought to target voltage-gated potassium channels (VGKC) (Paterson et al. 2014). Clinical studies using patients selected on the basis of anti-VGKC antibody positivity failed to select homogenous groups of patients (Klein et al. 2013; Malter et al. 2014). In addition, the definition of the Morvan syndrome is relatively vague, since it is based on old clinical descriptions dating back from the second half of the twentieth century (Roger, Alliez, and Roger 1953; Irani et al. 2012). Besides, until recently case series focusing on the distribution of the symptoms in the patients were scarce (Lancaster et al. 2011). Moreover, recent data suggest that some CASPR2-Abs patient have symptoms (such as ataxia, or hyperkinetic movement disorders) that do not fit into any of the three classical CASPR2-Abs syndromes(Becker et al. 2012; Balint et al. 2013; Gövert et al. 2016). Confusion in the clinical classification is an obstacle to studying disease mechanisms, and for getting better insight into the physiopathology of CASPR2-Abs diseases, it will be needed

first to have a clearer idea of the clinical presentations of the patients. Therefore, it is necessary to study cohorts of patients selected on the basis of CASPR2-Abs positivity and verify if the clinical patterns fit with the classical CASPR2-Abs syndromes. To guide this effort, it is useful to review the current concepts about CASPR2-Abs and the syndromes they associate with.

FROM ANTI-VGKC TO ANTI-CASPR2 ANTIBODIES

Originally, autoantibodies in patients with acquired neuromyotonia were detected using a radioimmunoassay in which neurotoxins derived from the venom of green mamba snakes were used to precipitate VGKCs from brain lysates (Shillito et al. 1995; Hart et al. 2002). Antibodies binding such precipitates were then considered to target the VGKCs, even though such precipitates also contain VGKC-interacting proteins (van Sonderen, Schreurs, et al. 2016). Over time, the clinical spectrum of the so-called anti-VGKC antibodies expanded, to include not only neuromyotonia, but also autoimmune limbic encephalitis, and Morvan syndrome (Cornelius et al. 2011; Shillito et al. 1995; A. Vincent et al. 2004). The detection process was later refined to restrict detection to specific subtypes of potassium channels (such as Kv1.1, Kv1.2, and Kv1.6) in an attempt to make correlations between the VGKC subunit targeted and the clinical presentation of the patients (Kleopa et al. 2006). Interestingly, such "anti-VGKC" antibodies purified from the patients' sera were shown to increase the excitability of cultured dorsal root ganglia neurons, and, when injected intraperitoneally in mice, to increase acetylcholine release at neuromuscular junction and increase peritoneal nerve action currents (Sinha et al. 1991; Shillito et al. 1995). Therefore, it was considered that patients with neuromyotonia or autoimmune limbic encephalitis had antibodies targeting different types of VGKC, altering potassium channels function thereby leading to the patients' symptoms (Kleopa et al. 2006). However, identification of anti-VGKC antibodies using the

much more specific cell-based binding assay is usually negative, suggesting the patients' antibodies do not directly target potassium channels, but to yet unknown VGKC-interacting proteins instead (Lancaster et al. 2011). From this observation, Lancaster and colleagues performed immunoprecipitation studies from anti-VGKC antibodies patients' sera and demonstrated that the actual target antigens were in fact LGI1 and CASPR2, two proteins involved in the regulation of VGKC expression (Lai et al. 2010; Lancaster et al. 2011). These antibodies appeared to be clinically relevant, since they associate with distinct clinical features, such as brachio-facial dystonic seizures and poorer memory outcomes in anti LGI1 antibodies patients, and neuromyotonia in CASPR2-Abs patients (Malter et al. 2014; S. N. M. Binks et al. 2018). Conversely, patients tested positive for anti-VGKC antibodies but negative for anti-LGI1 and anti-CASPR2 antibodies have antibodies that target intracellular components of the VGKC complex, and have non-immune disease etiologies (van Sonderen, Schreurs, et al. 2016; Lang et al. 2017). Such evidences eventually led to the general consensus that anti-VGKC antibodies should not be used anymore to screen patients suspected of autoimmune neurological disorders and the term was gradually abandoned in favor of anti-LGI1 and anti-CASPR2 antibodies (van Sonderen, Schreurs, et al. 2016).

AUTOIMMUNE LIMBIC ENCEPHALITIDES

Limbic encephalitis, first described in 1968, was initially considered strictly as a paraneoplastic neurological syndrome (Corsellis, Goldberg, and Norton 1968). Clinically, limbic encephalitis features symptoms of limbic dysfunction, including anterograde amnesia, temporal lobe seizures and behavioral changes (Gultekin et al. 2000). Morphologically, it is associated with mesiotemporal lobe inflammation (visible in brain MRI scan in the form of usually bilateral hippocampal T2-weighted hyperintensities) with prominent T-CD8+ cells

infiltrates and diffuse neuronal loss (Corsellis, Goldberg, and Norton 1968; Gultekin et al. 2000; Christian G. Bien et al. 2012).

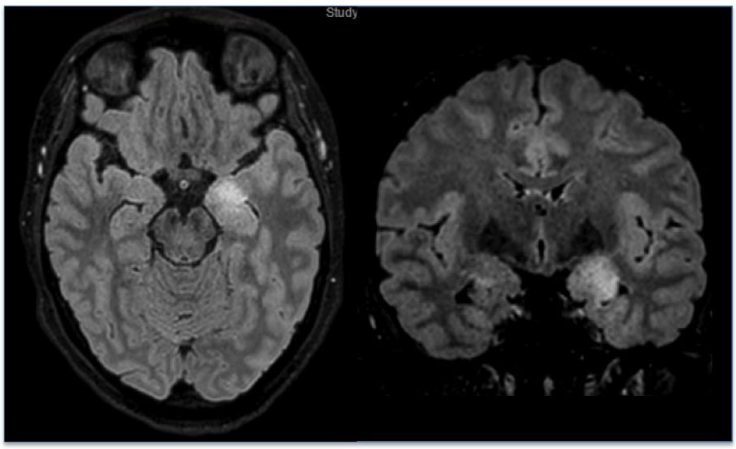

Figure I. Unilateral hippoampus and amygdala hyperintensity in a patient with autoimmune encephalitis and anti-LGI1 antibodies (Brain magnetic resonance imaging, T2-weighed sequences, axial (left), and coronal view (right))

Functional outcomes are classically poor, and most patients are left with severe memory loss (Gultekin et al. 2000; Graus et al. 2001). Paraneoplastic limbic encephalitis has been described in association with various cancers and onconeuronal antibodies (such as Hu, CV2/CRMP5, Ma2, etc.) (Gultekin et al. 2000; Graus et al. 2001; Honnorat et al. 2009; Dalmau et al. 2004). However, it later became clear that there were limbic encephalitis patients who did not have any detectable tumor, whose symptoms were not limited to limbic signs, and who had remarkably good response to immunotherapy (Bataller et al. 2007). In addition, these patients did not have onconeuronal antibodies but instead had antibodies targeting then yet unidentified proteins expressed at the cell surface of the neurons (Bataller et al. 2007). These clinical observations led to the description in 2007 of antibodies targeting the NMDA receptor in patients with a frequently non-paraneoplastic, treatable form of autoimmune limbic encephalitis (Sansing et al. 2007; Iizuka et al. 2008; Dalmau et al. 2007). This caused a complete paradigm switch of limbic encephalitis, and soon other antibodies

targeting neuronal cell-surface antigens were described in subgroups of limbic encephalitis patients, including anti-AMPAR, -$GABA_AR$ and -$GABA_BR$, and -DPPX antibodies (Lai 2009, Lai 2010, Lancaster 2011, Petit-Pedrol 2014, Höftberger 2013) (Table I). These entities were called autoimmune encephalitides, a group of autoimmune neurological diseases characterized by subacute neurological symptoms which prominent limbic affectation, autoantibodies targeting surface neuronal antigens, and good response to immunotherapies (Dalmau and Graus 2018) (Table I). Diagnostic criteria have been recently developed based on experts' consensus (Graus et al. 2016). Most autoimmune encephalitides are antibody-mediated disorders in which the binding of the antibodies onto their target, which are usually proteins involved in synaptic transmission, functionally impair synaptic transmission, but cause no or little neuronal loss (Planagumà et al. 2016; Petit-Pedrol et al. 2018; Haselmann et al. 2018) (Table I). CASPR2-Abs have been linked to autoimmune limbic encephalitis in the seminal paper from Lancaster and colleagues in 2010, but the number of patients reported at the time was too small to infer the clinical specificities of autoimmune encephalitis when associated with CASPR2-Abs (Lancaster et al. 2011).

Antibody Target	Number of published cases	Fonction of the targeted protein	Effect of autoantibodies	Associated tumors	Main clinical features	References
NMDAr	> 3000	Glutamate receptor; involved in synaptic plasticity	NMDAr internalization Disrupted interaction between NMDAr and Ephrin B2 at synapses	Ovarian teratoma in adult women (26-59%) Tumors are rare in men and children	Acute psychosis Seizures, focal temporal lobe, or tonic clonic Impaired consciousness Dysautonomia Oro-facial and segmental dyskinesia Speech impairment	(Dalmau et al. 2007; 2011; Titulaer et al. 2013)
LGI1	> 700	Secreted protein Modulates potassium channels and regulates glutamatergic transmission	Loss of LGI1-ADAM22 interaction	5% (lung, thymoma)	Brachiofacial dystonic seizures Temporal lobe epilepsy Anterograde amnesia Dysexecutive syndrome REM sleep behavioral disorders	(van Sonderen, Thijs, et al. 2016; Irani et al. 2013; Navarro et al. 2016)
IgLON5	≈90	Cell adhesion molecule	Internalization of IgLON5	Not reported	Sleep disorders Cognitive impairment Bulbar palsy Dysautonomia Movement disorders Cerebellar ataxia Oculomotor symptoms	(Sabater et al. 2014; 2016)
GAD65	60	Synthesis of GABA	unknown	Rare	Drug-resistant temporal lobe epilepsy Memory impairment Chronic progression	(Saiz et al. 2008; Ariño et al. 2015)
AMPAr	≈ 60	Glutamate receptor; synaptic plasticity	Internalization of AMPAr	50- 60% of the patients (lung, thymoma, breast)	Variable: limbic encephalitis, panencephalitis, ataxia	(Lai et al. 2009; Joubert et al. 2015; Höftberger et al. 2015)

$GABA_AR$	≈ 50	Ionotropic GABA receptor	Unknown	40% (thymoma)	Mostly affects children Temporal lobe and generalized epilepsy Memory impairment Behavioral disorders	(Petit-Pedrol et al. 2014; Spatola et al. 2017)
$GABA_BR$	≈150	Metabotropic GABA receptor	Unknown	60-100% (small-cell lung cancer)	Focal temporal lobe and generalized epilepsy Severe cognitive impairment	(Maureille et al. 2019; van Coevorden-Hameete et al. 2019)
DPPX	40	Involved in potassium channels assembly	DPPX internalization	Rarely, haematological cancers	Psychiatric symptoms Abnormal movements Diarrhea, weight loss	(Balint et al. 2014; Hara et al. 2017)
mGluR5	15	Metabotropic glutamate receptor	Decreased surface expression of mGluR5	50% (Hodgkin lymphoma)	Psychiatric symptoms Temporal lobe seizures Memory impairment	(Spatola et al. 2018, 5)
AK5	15	intracellular neuronal protein	Unknown	Not reported	Massive anterograde amnesia	(Do et al. 2017; Ng et al. 2015, 5)
Neurexin-3α	6	Cell-adhesion protein; synapse fomation and maintenance	Internalization of neurexin-3α ; Decreased number of synapses	Not reported	Tonic-clonic generalized seizures Confusion Impaired vigilance Memory impairment	(Gresa-Arribas et al. 2016)

Table I. Main types of autoimmunes encephalitides: associated antibodies, known pathophysiology, and clinical features.

ACQUIRED NEUROMYOTONIA

Acquired neuromyotonia, or Isaacs' syndrome, is a syndrome of spontaneous and continuous muscular fiber activity (Newsom-Davis and Mills 1993). Clinically, acquired neuromyotonia is characterized by positive motor symptoms (fasciculations, myokimia, cramps, limb stiffness), neuralgic pain (usually with a length-dependant distribution), and autonomic symptoms (hyperhidrosis, orthostatic hypotension, constipation). Electroneuromyographic recordings reveal signs of peripheral nerve hyperactivity, in form of fasciculations, multiplets, post-discharge activity, and myokimic and neuromyotonic discharges (Newsom-Davis and Mills 1993). Forms with milder presentations and needle electromyogram showing only fasciculation potentials are sometimes labeled as cramp fasciculation syndrome (Sawlani and Katirji 2017). Although some inborn neurological syndromes can include symptoms of peripheral nerve hyperexcitability, most cases are acquired (Peeters et al. 2017). Causes include toxic, immunoallergic, and above all autoimmune affections (Sawlani and Katirji 2017). A few of the reported acquired neuromyotonia patients had a concomitant thymoma, but most reported cases are not paraneoplastic (Gastaldi et al. 2019; Fleisher et al. 2013; A. Vincent et al. 2018). Evidences suggesting an autoimmune basis include the association with other autoimmune diseases, the rare cases induced by penicillamine treatment, and the occasional association with thymoma (Reeback et al. 1979; Fleisher et al. 2013; A. Vincent et al. 2018; Gastaldi et al. 2019). Acquired neuromyotonia was linked to autoimmune processes when it was shown that the patients' serum contain neuron-binding immunoglobulins, that were later identified as anti-VGKC antibodies (Shillito et al. 1995). In parallel, application on cultured neurons of serum from neuromyotonia patients increased neuronal excitability and the injection of patients purified immunoglobulins in mice were associated with increased acetylcholine release at muscular endplates and increased peroneal nerve action currents, suggesting peripheral nerve hyperexcitability is antibody-mediated (Shillito et al. 1995). The

association with CASPR2-Abs was formally demonstrated only in 2011 (Lancaster et al. 2011). However, >70% of the cases are not associated with CASPR2-Abs, suggesting the spectrum of acquired neuromyotonia is much broader (A. Vincent et al. 2018). GABAergic treatments and antiepileptic drugs are usually beneficial as well as immunotherapies (Sawlani and Katirji 2017).

MORVAN SYNDROME

In 1890, the French physician Augustin Morvan described six patients with generalized "fibrillar contractions of the muscles" and pain. The clinical description he made matches that of acquired neuromyotonia, except for one of the patients who had marked deterioration of the general status, confusion, insomnia, profuse hypersudation and tachycardia (Morvan 1890; Walusinski and Honnorat 2013). In 1953 Roger and colleagues reported a series of 70 cases and delineated the clinical features of what they called the Morvan syndrome (Roger, Alliez, and Roger 1953). According to these authors, Morvan syndrome features peripheral nerve hyperexcitability symptoms (similar to Isaacs syndrome) along with severe dysautonomia, confusion, and insomnia. The autonomic symptoms are particularly intense and are predominated by profuse hypersudation (classically patients need to change clothes or bed sheets several times a day) and cardiovascular manifestations (sinusal tachycardia, pseudo-erythromelalgia, blood pressure fluctuations). Insomnia is extremely severe and has been qualified with the term agrypnia excitata, a rare polysomnographic pattern initially described in fatal familial insomnia patients (Lugaresi and Provini 2001). Agrypnia excitata consists of a complete loss of slow-wave sleep while short periods of REM sleep remains, interspersed with awakenings. Complex visuo-auditory hallucinations occur at night, possibly due to the quickly alternating awake and REM-sleep states (Lugaresi and Provini 2001). Soon after CASPR2-Abs were described, it appeared that part of the CASPR2-Abs patients had a

clinical presentation compatible with the Morvan syndrome (Irani et al. 2012). However, only one Morvan syndrome cohort has been published since the identification of CASPR2-Abs, and in this publication, the diagnosis of Morvan syndrome was considered whenever a patient had a combination of neuromyotonia and encephalopathy (Irani et al. 2012). This series emphasized the male predominance, the importance of autonomic symptoms, neurogenic pain, sleep disorders, and weight loss, as well as the frequent association with thymoma and myasthenia gravis. Interestingly, half of the patients had dual antibody positivity (LGI1/CASPR2), some patients were negative for CASPR2-Abs but had LGI1-Abs, and a few patients had antibodies targeting yet unknown neuron-surface antigens. Yet, the definition of Morvan syndrome in the literature is confused. For one part, some authors consider that Morvan syndrome represents merely the association of acquired neuromyotonia and limbic encephalitis, and is not a distinctive syndrome (Irani et al. 2012). On the other hand, some authors consider Morvan syndrome as a genuine entity in which agrypnia, autonomic symtptoms, and peripheral nerve hyperexcitability are prominent features (Laurencin et al. 2015). In addition, the prognosis of the Morvan syndrome remains unclear. In the article by Irani and colleagues, outcomes were poor because many patients had general complications (aspiration pneumonia, sepsis, cardiac arrest) (Irani et al. 2012). By contrast, isolated case reports suggest that patients improve dramatically with rituximab (Sadnicka et al. 2011; Ong et al. 2013; Weiss 2012).

EXPRESSION AND FUNCTIONS OF CASPR2

Structure of CASPR2.

CASPR2 is a neuroglial cell-adhesion protein belonging to the neurexin superfamily (Poliak et al. 1999). Most neurexins are pre-synaptic proteins involved in the formation of excitatory and inhibitory synapses through interaction with post-synaptic neuroligins (Graf et al. 2004). Interestingly, autoantibodies targeting the neurexin-3α have been found in patients with a subtype of autoimmune encephalitis, and anti-neurexin-3α antibodies from the patients' serum alters synapse development *in vitro* (Gresa-Arribas et al. 2016). CASPR2 for its part is found in a great variety of neurons and is expressed in the adult rodent brain in multiple brain areas related to motor activity (such as basal ganglia and pontine nuclei), in limbic structures (e.g. hippocampus, amygdala), and in sensory pathways (e.g. dorsal part of the spinal cord, dorsal root ganglia) (Poliak et al. 1999; Gordon et al. 2016). CASPR2 is a transmembrane protein with an intracellular region containing a 4.1B binding motif and a PDZ-binding motif (Horresh et al. 2008; Denisenko-Nehrbass et al. 2003). Its extracellular part is composed of an N-terminal discoidin-like domain, four laminin G-like domaines, two epidermal-like growth factor domains, and a fibrinogen-like domain (Poliak et al. 1999) (Figure IV). Although the aminoacidic sequence of CASPR2 is very close to that of neurexin-1α, the discoidin-like and most distal fibrinogen-like domains are specific to CASPR2 (Poliak et al. 1999). Moreover, CASPR2 has a compact organization while neurexin-1α has an elongated conformation (Lu et al. 2016; Rubio-Marrero et al. 2016). CASPR2 extracellular domain is organized into 3 lobes that can take two main conformations, vertical or horizontal (Lu et al. 2016). Such conformational changes may be involved in modulating the ability to bind protein partners or its addressing to specific cellular subregions (Figure IV). In addition, CASPR2 is part of multi-protein complexes that include proteins such as TAG-1 (or contactin-2), LGI1, the

metalloproteases ADAM 22 and ADAM 23, the VGKC subunits Kv1.1 and Kv1.2, and Kvβ2, and the intracellular proteins PSD-93, PSD-95, 4.1B protein, MMP2, and MUPP-1 (multiple PDZ domain protein-1) (Inda, DeFelipe, and Muñoz 2006; Duflocq et al. 2011; Chen et al. 2015; Pinatel et al. 2017; Tanabe et al. 2015, 201) (Table II). The composition of this complex may change according to subcellular location, meaning co-immunoprecipitation studies from whole brain extracts are difficult to make sense of (Chen et al. 2015).

In pyramidal neuronal cultures, CASPR2 is found expressed at both soma and dendrites, although over time a redistribution of surface CASPR2 with preferential axonal localization is observed (Poliak et al. 1999; Bel et al. 2009; Pinatel et al. 2015). This redistribution is dependent on a selective endocytosis process mediated by the binding to the intracellular protein 4.1B (Bel et al. 2009). Cultures of hippocampal neurons show that CASPR2 is expressed only by a fraction of excitatory and inhibitory neurons, in proportions that vary among studies (Varea et al. 2015; Pinatel et al. 2015; Vogt et al. 2018; Fernandes et al. 2019). In addition, CASPR2 colocalizes with Kv1 voltage-gated potassium channels at two specific axonal microdomains, the juxtaparanodes, and the axonal initial segment (AIS) (Ogawa et al. 2008). Since the Kv1 potassium channels have a fundamental role in repolarising the axon after action potential initiation, at the AIS and JXPN regions as well as in dendritic spines, that CASPR2 colocalizes with Kv1 channels at such strategic subdomains suggests a role in regulating neuronal excitability (Trimmer 2015).

Protein	Type	Interacting domains on CASPR2	Role	References
TAG-1	GPI-anchored protein	laminin G1, EGF2 and laminin G4 domains	Interaction with CASPR2 in CIS and TRANS, recruitment of Kv1 channels at juxtaparanodes	(Poliak et al. 2003; Saint-Martin et al. 2019)
Kv1.1/Kv1.2/ Kvβ2	Voltage-gated potassium channel	No direct interaction	Regulation of axon potential propagation, repolarisation after axon potential initiation	(Trimmer 2015)
ADAM-22	Membrane-anchored metalloprotease	CASPR2 ectodomain	MAGUK recruitment to juxtaparanode	(Ogawa et al. 2010)
ADAM-23	Membrane-anchored metalloprotease	CASPR2 ectodomain	Involved in TAG-1 and CASPR2 addressing along axonal microdomains	(Hivert et al. 2019)
LGI1	Secreted synaptic protein	No direct interaction	regulation of glutamatergic synapses excitability, expressed at the AIS	(Hivert et al. 2019)
4.1B protein	Protein 4.1 family	4.1B binding domain	Recruitment of Kv1 channels at juxtaparanodes by interacting with CASPR2	(Traka et al. 2003)
PSD-93	MAGUK	No direct interaction	Kv1 clustering at the axon initial segment independantly of CASPR2	(Horresh et al. 2008; Pinatel et al. 2017)
PSD-95	MAGUK	No direct interaction	Enriched at the JXP, but no role in Kv1 recruitment	(Rasband et al. 2002)
MMP2	MAGUK	PDZ type II domain	Unknown, enriched at the AIS	(Pinatel et al. 2017)
MUPP-1	Scaffolding protein	PDZ type II domain +/- 4.1B binding domain	Colocalizes with CASPR2 and GPR37 at the synapse of cultured hippocampal neurons	(Horresh et al. 2008; Tanabe et al. 2015)

Abbreviations:
GPI: glycosyl phospahtidyl-inositol
MAGUK: membrane-associated guanylate kinase
JXP: juxtaparanode
AIS: axon initial segment

Table II. Proteins found in CASPR2-containing macromolecular complexes

CASPR2 at the juxtaparanodes

The JXP are finely regulated regions that flank the nodes of Ranvier, and are characterized by the presence of a macromolecular complex that includes CASPR2, TAG-1 (Transient Axonal Glycoprotein-1) 4.1B protein, and the Kv1.1/1.2 channel (Poliak et al. 2003; Traka et al. 2003). Kv1 channels expression at the JXP is important to regulate axonal potential propagation along myelinated axons (Trimmer 2015) and their recruitment at the JXP requires the of interaction of CASPR2, TAG-1 and the intracellular 4.1B protein (Poliak et al. 2003; Traka et al. 2003). Accordingly, the knockout of CASPR2 in mice cause impairs recruitment of Kv1 at the JXP and increases excitatory post-synaptic responses (Scott et al. 2019). TAG-1, also known as Contactin-2 or Axonin-1, a GPI-anchored cell-surface protein of the immunoglobulin superfamily is found expressed in both axons and glial cells, is also necessary for Kv1 recruitment at the JXP, where it interacts with CASPR2 in CIS and in TRANS (Poliak et al. 2003; Traka et al. 2003). Importantly, although PSD-93 and PSD-95 are found in the JXP macromolecular complex, they do not interact directly with CASPR2 and do not drive CASPR2 and Kv1 clustering at the JXP (Horresh et al. 2008). Conversely, 4.1B protein binding to the intracellular region of CASPR2 is necessary for the JXP accumulation of CASPR2, TAG-1, Kv1 and PSD-93 (Horresh et al. 2010; Cifuentes-Diaz et al. 2011; Buttermore et al. 2011). Overall, CASPR2 localization at the JXP is driven by 4.1B protein, where it interacts with TAG-1 to recruit Kv1 channels, thereby promoting the proper organization of the JXP (Figure II).

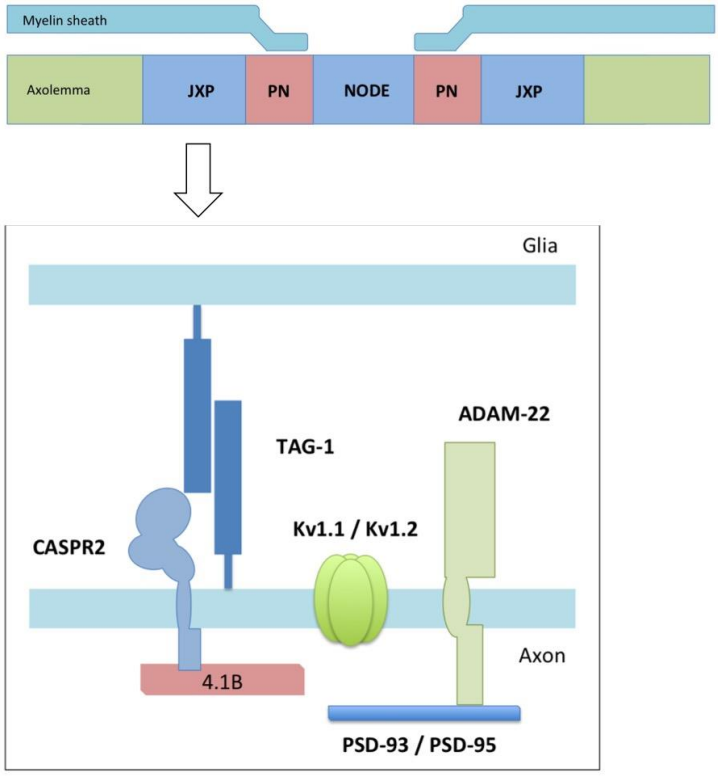

Figure II. Schematic organization of the CASPR2 macromolecular complex at the JXP. CASPR2 enrichment at the JXP depends on its interaction with the 4.1B protein. CASPR2 interacts in CIS and TRANS with TAG-1, thereby promoting the recruitment of Kv1.1/1.2 channels. ADAM-22 colocalizes with CASPR2 but is involved only in the recruitment of PSD-93 and PSD-95, which are not necessary for CASPR2 or Kv1 channels recruitment.
Abbreviations. JXP: juxtaparanode, PN: paranode.

CASPR2 at the axon initial segment

In addition to the JXP, CASPR2 colocalizes with TAG-1 and the Kv1 channels at the axonal initial segment (AIS), a specialized region of the neurons where the action potential is generated (Inda, DeFelipe, and Muñoz 2006; Duflocq et al. 2011). Protein surface expression is finely regulated at the AIS, with an important role for the scaffold protein AnkyrinG (Pinatel et al. 2015). Of note, CASPR2 was also reported at a specific subcellular region adjacent to the AIS, the juxtapara-AIS (JXP-AIS), which is organized similarly to the JXP and is analogous to a hemi-node placed at the initial part of the myelin sheath (Duflocq et al.

2011). Surprisingly, even though CASPR2 colocalizes at the AIS with TAG-1 and Kv1.1 and 1.2 channels, it is evenly distributed along the axon and is only poorly enriched at the AIS (Pinatel et al. 2017) (Figure III). By contrast, TAG-1 is strongly enriched at the AIS, although the proteins that drive this selective targeting are unknown (Pinatel et al. 2017). Furthermore, the recruitment of Kv1 channels at the AIS does not depend on CASPR2 or TAG-1, but on the PSD-93 expression instead (Inda, DeFelipe, and Muñoz 2006; Ogawa et al. 2008; 2010; Duflocq et al. 2011). Accordingly, the 4.1B protein, which is necessary for CASPR2 redistribution and targeting to the JXP, is not enriched at the AIS (Duflocq et al. 2011). Interestingly, a study using CASPR2 constructs showed that the ectodomain of CASPR2 contains determinants for distal axon distribution, while its intracellular tail contains determinants targeting to the AIS (Pinatel et al. 2017). Therefore, the differential distribution of CASPR2 and TAG-1 along the axon may rely on selective partnerships with yet unknown proteins and on conformational changes of the CASPR2 ectodomain (Pinatel et al. 2017) (Figure III). Overall, the determinants and roles of CASPR2 outside the JXP are still poorly known.

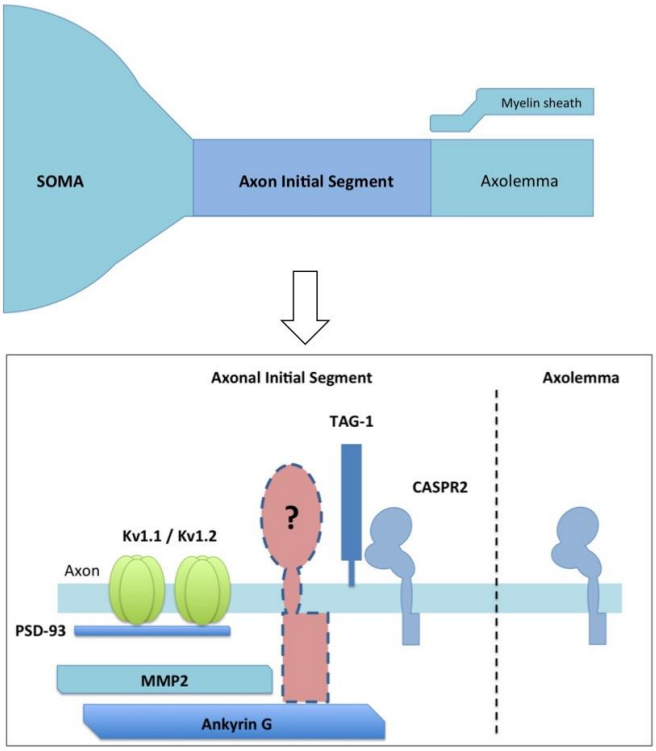

Figure III. Schematic organization of the CASPR2 macromolecular complex at the JXP. CASPR2 colocalizes at the AIS with TAG-1 and the Kv1 channels, but is only poorly enriched at this site compared to the rest of the axolemma. By contrast, TAG-1 is enriched at the AIS, likely through the interaction with yet unidentified neuron-surface proteins. Kv1 channel recruitment at the AIS depends on PSD-93 instead of CASPR2/TAG-1. Scaffold proteins MMP2 and Ankyrin, while enriched at the AIS, do not directly interact with CASPR2.

Other subcellular localizations of CASPR2.

Surprisingly, CASPR2 is not found at the basket cell terminals, where Kv1.1/1.2 channels expression is critical for cerebellar functions (Ogawa et al. 2010). However, CASPR2 expression in respect to Kv1 channels has not been studied in the cerebellar deep nuclei, where Kv1.1 channels are important for the regulation of cerebellar outputs (Ovsepian et al. 2013). In addition, the degree of expression of CASPR2 in synapses remains unclear. Subcellular fractioning studies found CASPR2 in the synaptosomes and in synaptic membrane fractions (Chen et al. 2015; Bakkaloglu et al. 2008). Immunofluorescence studies using cultures of neurons suggest that CASPR2 can be found at both glutamatergic and

GABAergic synapses, although it is unclear if it is preferentially pre- or post-synaptic (Varea et al. 2015; Pinatel et al. 2015; Vogt et al. 2018; Bonetto et al. 2019; Fernandes et al. 2019). In addition, knockout studies have suggested that CASPR2 is involved in AMPA receptors turnover at the synapse and in GABAergic signaling (Varea et al. 2015; Pinatel et al. 2015; Vogt et al. 2018; Fernandes et al. 2019).

Roles of CASPR2 in the developing nervous system.

Mutational and knockout studies of CASPR2 in mice has shown a critical role in the developing nervous system for axonal growth, dendritic arborization, and spine development (Anderson et al. 2012; Canali et al. 2018). In addition, mutations or knockout of CASPR2 delays myelination in the neocortex, but not in white matter tracts or peripheral nerves (Poliak et al. 2003; Scott et al. 2019). CASPR2 expression is important as well for the development of cortical GABAergic interneurons and therefore for the establishment of proper excitatory/inhibitory balance in the cortex of the developing brain (Vogt et al. 2018; Selimbeyoglu et al. 2017). Other authors, by contrast, found a prominent alteration of post-synaptic excitatory responses and not in inhibitory post-synaptic currents (Scott et al. 2019). In accordance with these developmental functions, mutations of CASPR2 have been associated with various neurodevelopmental and psychiatric abnormalities that include autistic-spectrum disorders, cortical dysplasia with focal epilepsy, intellectual disability, attention deficit hyperactivity disorder, obsessional compulsive disorder, and Gilles de la Tourette syndrome (Bakkaloglu et al. 2008; Poot 2017). The variety of observed phenotypes highlights the multiples roles played by CASPR2 in the mature and in the developing nervous system.

EFFECTS OF ANTI-CASPR2 ANTIBODIES

Several studies have focused on the effects that CASPR2-Abs from encephalitis or neuromyotonia patients may have on neuronal function, but obtained conflicting results. For instance, Fernandes and colleagues reported that incubation of cultured hippocampal neurons with purified serum immunoglobulins from anti-CASPR2 antibody-positive patients induces fast internalization of both CASPR2 and AMPAR on cultured neurons, while using similar models Patterson et al and Saint-Martin et al found no change in surface CASPR2 expression (Fernandes et al. 2019; Patterson, Dalmau, and Lancaster 2018; Saint-Martin et al. 2019). In one study using cultured dorsal root ganglia neurons, CASPR2-abs incubation resulted in a decrease of Kv1 channels, while Saint-Martin et al found an increase in Kv1 neuronal expression following treatment of hippocampal neurons with CASPR2-Abs (Dawes et al. 2018; Saint-Martin et al. 2019). However, two groups have showed independently that CASPR2-Abs are able to block the interaction between TAG-1 and CASPR2 (Patterson, Dalmau, and Lancaster 2018; Saint-Martin et al. 2019). Interestingly, CASPR2-Abs targets only one of the two domains involved in the interaction with TAG-1 (the laminin G1 but not the laminin G4 domain), suggesting the lack of interaction is due to antibody-induced conformational change (Saint-Martin et al. 2019) (Figure IV).

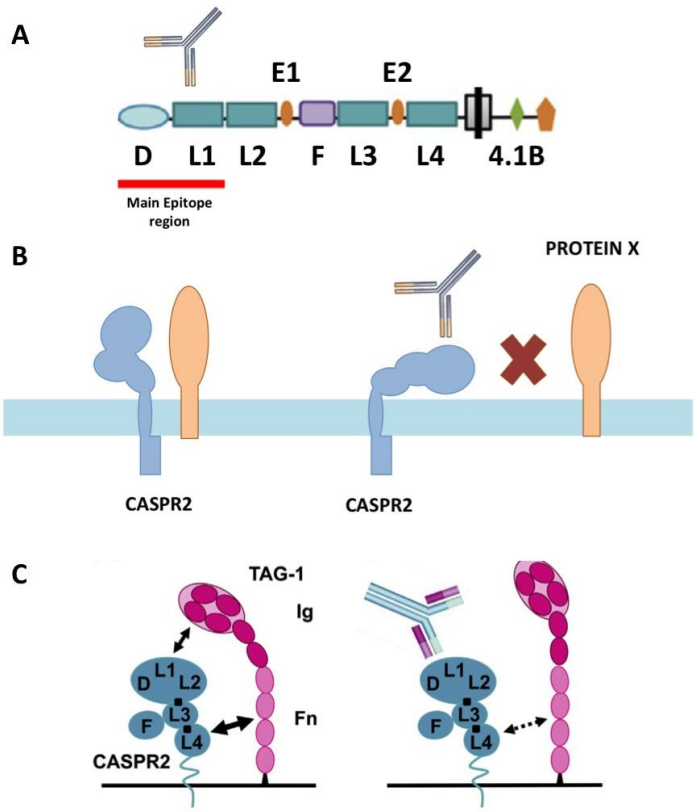

Figure IV. Putative effects of CASPR2-Abs on CASPR2 structure and function.
CASPR2-Abs preferentially bind epitopes on the discoidin and laminin G1 domains of CASPR2 (A). Since CASPR2 can present with two different conformations (horizontal or vertical) that may influence the ability to bind partner proteins, CASPR2-Abs binding may favor a conformation over another, thereby impeding binding to partner proteins (B). Supporting this hypothesis, Saint Martin et al. found that CASPR2-Abs impedes interaction with TAG-1 without targeting the main site for TAG-1 binding on CASPR2 (Saint-Martin et al. 2019) (C).

Of note, CASPR2-Abs are mostly of the IgG4 isotype, which lacks covalent liaisons between heavy chains, and is therefore bispecific (Schuurman et al. 1999). Whether CASPR2-Abs in the patients are actually bispecific (e.g. targeting two different epitopes on CASPR2), and whether bispecificity plays a role in their effect on CASPR2 binding to TAG-1, remain unclear. Interestingly, intraperitoneal injection in mice of purified immunoglobulins from anti-CASPR2 antibody-positive patients lowers the nociceptive thresholds of the animals and decreases Kv1 and CASPR2 expression at the juxtaparanodes in the sciatic nerves and at the

surface of dorsal root ganglia neurons (Dawes et al. 2018). As patients antibodies were found to bind the dorsal root ganglia neurons of the animals, but not to reach the juxtaparanodes, the authors proposed that antibody-mediated CASPR2 dysfunction at the level of the dorsal root ganglia result in widespread downregulation of CASPR2 and Kv1 channels, ultimately affecting juxtaparanodal organization (Dawes et al. 2018). Besides these putative peripheral effects, there have been an attempt of CASPR2 encephalitis mouse model, using intraperitonal injections of CASPR2-Abs followed by LPS-induced blood brain barrier opening, but it failed to obtain any conclusive result (Giannoccaro et al. 2019). Apart from the effects of the antibodies, the immunological processes leading to the break of immune tolerance for CASPR2 are unknown. Paraneoplastic cases with malignant thymoma have been reported, suggesting involvement of aberrant thymocyte maturation in anti-CASPR2 autoimmunity (A. Frcp. Vincent and Irani 2010). On the other hand, association with the HLA-II DRB1*1101 haplotype have been shown, suggesting genetic determinants of the immune response are involved in the development of the disease (S. N. M. Binks et al. 2018). Likely, the combination of multiple factors is involved.

OBJECTIVES OF THE PHD PROJECT

The clinical spectrum of CASPR2-Abs remains incompletely understood. First, it is unclear if all patients fit into one of the three classical CASPR2-Abs syndromes mentioned above, that is, autoimmune limbic encephalitis, acquired neuromyotonia, and Morvan syndrome. Second, the clinical specificities of autoimmune limbic encephalitis associated with CASPR2-Abs was not comprehensively studied in the first published series of CASPR2-Abs patients, and more recent publications suggest it may be not restricted to the limbic symptoms. In particular, the importance of hyperkinetic movement disorders and cerebellar ataxia remains to be assessed (Klein et al. 2013; Balint et al. 2013). In addition, the definition of Morvan syndrome remains imprecise, and there is only one case series from the modern era that can help to better define this syndrome (Irani et al. 2012). Last, the immunological and oncological associations that may influence neurological phenotype remain unknown. Improving the classification of CASPR2-Abs patients is of particular importance since a better understanding of CASPR2-Abs associated disease is necessary for planning future studies focusing on the physiopathology of the disease. In addition, refining our knowledge of CASPR2-Ab syndromes may promote early identification of the patients and avoid delays in diagnosis and treatment. It is also important for the management of the patients to have a clearer idea of what outcomes and responses to treatment are to expect.

Accordingly, this PhD project aims at improving the clinical classifications of the patients, using a French nationwide retrospective cohort of CASPR2-Abs patients for which clinical data and biological samples - including serum, cerebrospinal fluid (CSF), and DNA - are available. Our objectives are the following:

1. To study symptoms' distributions in the patients in order to identify clinical patterns;

2. To identify patients with a clinical presentation compatible with either autoimmune limbic encephalitis or Morvan syndrome, and to study clinical presentation and outcomes;

3. To analyze correlations between clinical patterns and antibody specificities, cancer associations and HLA phenotypes.

The present PhD project is divided into three studies. The first study focuses on the immunological and clinical features of patients with CASPR2-Abs and autoimmune limbic encephalitis (Joubert et al. 2016). The second study reports episodes of transient cerebellar ataxia in patients with CASPR2-Abs (Joubert et al. 2017). The third study uses statistical methods to analyze the distribution of the clinical symptoms of the patients, in order to assess if different groups of patients can be distinguished according to patterns of clinical presentation (Muñiz-Castrillo et al., submitted for publication, under review).

CLINICAL STUDIES

FIRST STUDY

PRESENTATIONS AND OUTCOMES IN CASPR2 ENCEPHALITIS

Cerebrospinal fluid anti-CASPR2 antibodies determine a subtype of autoimmune encephalitis with prominent limbic symptoms and seizures.

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ABSTRACT

Importance. Autoantibodies against CASPR2 are observed in several neurological syndromes, including neuromyotonia, Morvan's syndrome and limbic encephalitis.

Objective. To characterize the clinical and biological presentations of patients with anti-CASPR2 antibodies in the cerebrospinal fluid (CSF).

Design. Retrospective analysis of patients with CSF anti-CASPR2 antibodies between March 2009 and November 2015.

Setting. Centre National de Référence pour les Syndromes Neurologiques Paranéoplasiques, Lyon, France.

Participants. Eighteen patients with CSF anti-CASPR2 antibodies compared to 15 patients with neuromyotonia or Morvan's syndrome.

Main outcomes and measures. Clinical presentations, anti-CASPR2 antibodies specificities, brain MRI and cerebrospinal fluid analyses, cancer prevalence, and evolution.

Results. Seventeen of the patients (94%) were male (median age, 64.5 years). Only three patients (17%) had a previous or concomitant history of cancer (prostate, haematological, and thyroid). Anti-CASPR2 antibodies were detected in the serum but not in the CSF of all neuromyotonia or Morvan's syndrome patients. A malignant thymoma was found in 9/15 (60%) of neuromyotonia or Morvan's syndrome patients and in none of the patients with CSF anti-CASPR2 antibodies. Symptoms of limbic encephalitis were observed in all patients, including temporal lobe seizures (16/18, 89%) and memory disorders (17/18, 94%). Extra-limbic signs were also evident in 12/18 patients (67%), including cerebellar ataxia in 6 patients (33%). Only two patients (11%) had neuromyotonia. Brain MRI displayed T2-weighted temporo-limbic abnormalities in 14/15 (93%) patients. CSF analysis was abnormal in 9/12 (75%)

patients. For 16 patients (89%), follow-up was performed for at least a 6-month period (median, 34 months). Fifteen patients (94%) improved and 6 of them (37.5%) relapsed. In all patients, CSF IgG4 autoantibodies were detected. CSF anti-CASPR2 antibodies targeted the laminin G1 and discoidin domains of CASPR2 in all patients.

Conclusions and relevance. CSF anti-CASPR2 antibodies associate with a subtype of autoimmune encephalitis, with prominent limbic involvement and seizures, that is rarely associated with cancer. Conversely, neuromyotonia and Morvan's syndrome patients have anti-CASPR2 antibodies only in the serum but not in the CSF and frequently present with a malignant thymoma. The CSF CASPR2 antibodies found in our patients targeted the discoidin and laminin G1 domains of CASPR2 and always included IgG4 autoantibodies.

INTRODUCTION

Antibodies directed against Contactin-associated protein 2 (CASPR2) have been described in the sera of patients with peripheral and central neurological syndromes, including neuromyotonia, Morvan's syndrome and autoimmune limbic encephalitis(Irani et al. 2010). Anti-CASPR2 antibodies (CASPR2 ab) belong to the anti-VGKC antibody complex, a biomarker that has been shown to include autoantibodies targeting CASPR2, Leucin-rich glioma 1 protein (Lgi1) and other unknown antigens(Malter et al. 2014). In addition, CASPR2 antibody-related syndromes have been frequently described in patients with malignant thymoma, a potential source of impaired T-cell maturation that may lead to autoantibody production(Irani et al. 2012; Laurencin et al. 2015). Although CASPR2 is involved in the organization of the juxtaparanodal regions of myelinated nerves, it is also expressed in the axons of hippocampal cells(Poliak et al. 2003; Bel et al. 2009). A recent study has suggested that CASPR2 ab from the cerebrospinal fluid (CSF) of limbic encephalitis patients bind to hippocampal inhibitory interneurons at the pre-synaptic level and have a disruptive effect on inhibitory synapses(Pinatel et al. 2015). Moreover, serum and CSF CASPR2 ab have been shown to be predominantly of the immunoglobulin G (IgG) isotype 4 (IgG4) and to target multiple extracellular epitopes of CASPR2, including its discoidin and laminin G1 domains(Pinatel et al. 2015; Olsen et al. 2015). Therefore, the diversity of CASPR2 antibody-associated diseases may rely on the implication of various factors, including the site of autoantibody synthesis, the epitope specificities of CASPR2 ab or the IgG subtypes involved. To further characterize patients with CASPR2 ab, we analyzed clinical and biological data from patients who were positive for CASPR2 ab in the CSF.

METHODS

In this study, we retrospectively included all patients for whom CSF samples were discovered positive for CASPR2 ab at the Centre de Référence National pour les Syndromes Neurologiques Paranéoplasiques (Lyon, France) between March 2009 and November 2015. During this period of time, we tested 6,650 CSF samples from French hospitals sent for routine diagnosis workout in patients suspected of autoimmune encephalitis or paraneoplastic neurological disorders. CSF samples of the patients were screened by immuno-histo-fluorescence on rat brain sections and CASPR2 ab were subsequently identified by a cell-based binding assay (CBA). In addition, serum samples of patients with confirmed CSF CASPR2 ab were screened for CASPR2 ab using a CBA. Written consent was obtained from all patients with approval of the institutional review board of the Hospices Civils de Lyon. Serum and CSF samples were deposited in the *NeuroBioTec biobank (Hospices Civils de Lyon, France, n° 0033-00046, AC-2013-1867, NFS96-900)*. Clinical data were collected from the referring physicians by phone and email. We focused on the modes of onset, clinical symptoms, ancillary results, therapy regimens, cancer prevalence and outcomes. In addition, patients diagnosed in our centre with neuromyotonia or Morvan' syndrome were tested for CASPR2 ab by CBA in both serum and CSF and were used as a comparative group. Age between groups was compared by Mann-Whitney U test. Sex ratio, Serum, CSF and co-morbidities between groups were compared using the Fisher exact test. Statistical significance was defined as p value less than 0.05.

For immuno-histo-fluorescence assays, sagittal slices obtained from adult rat brains were incubated with patient CSF samples (diluted 1:10) overnight at 4°C. Brain were fixed by immersion into paraformaldehyde 4% for 1 hour followed by immersion in a 30% sucrose solution during 24 hours. Histological slices were produced using a cryostat-microtome (Leica). Tissues were subsequently washed and incubated with

488-alexafluor-conjugated anti-human IgG (goat, Thermofisher, n°A21433). For the CBA, cultured HEK293 cells were transfected using the Lipofectamine LTX kit (Invitrogen) with plasmids coding for full-length or deleted CASPR2 fused to an influenza virus hemagglutinin tag (CASPR2-HA). Cells were incubated 48 hours post-transfection with CSF (diluted 1:10) or sera (diluted 1:20) and an anti-HA antibody (diluted 1:1000, Sigma-Aldrich, n°H3663) in DMEM, 25 mM HEPES, 3% Bovine Serum Albumin, 5% normal goat serum for 2 hours. Cells were subsequently washed in DMEM and HEPES during 15 minutes and incubated with alexafluor-conjugated secondary antibodies at 1:1000 dilution: goat anti-human total IgG-555 or -488; anti-mouse IgG-555 or -488 (Thermofisher, n° A21433, A11013, A21424 and A11029, respectively), mouse anti-human IgG1-488 or mouse anti-human IgG4-488 (Sigma-Aldrich, n° F9890 and F0767, respectively). Bound antibodies were visualized using a fluorescence microscope (Axiophot, Zeiss). CASPR2 ab titers in patient sera or CSF were assessed by serial 2-fold dilutions in CBA. Titers were determined as the lowest dilution that gave a positive signal, according to blinded investigators (VR, MSM). The epitopes targeted by the auto-antibodies of the patients were examined on CBA using CASPR2-HA deleted constructs as previously described(Pinatel et al. 2015) with the same dilutions (CSF, 1:10; serum, 1:20). Two blinded investigators (VR, MSM) assessed epitope and isotype specificities of CASPR2 ab. The IgG and albumin concentrations in the CSF and serum were evaluated using nephelometry (IMMAGE Immunochemistry Systems; Beckman-Coulter, Hialeah, FL). The CSF/serum albumin quotient (QAlb) was used to evaluate the integrity of the CSF–blood barrier. To characterize intrathecal immunoglobulin synthesis, the immunoglobulin quotient (QIg = [IgCSF/Igserum]) was calculated and adjusted to the corresponding QLim to quantify the CSF/serum CASPR2 ab index. QLim represents the maximum QIg that can be expected at a given QAlb in the absence of

intrathecal immunoglobulin synthesis(Reiber and Lange 1991). An antibody index greater than 4.0 was considered to reflect intrathecal synthesis(Reiber and Lange 1991).

RESULTS

Clinical findings. Among the 6,650 CSF samples tested in the French Reference Centre between 2009 and 2015 by immunohistochemistry, we identified 18 patients with CSF CASPR2 ab. During the same period of time, we identified 15 patients with serum CASPR2 ab and neuromyotonia or Morvan'syndrome (NMT/MoS). CSF samples from all of these NMT/MoS patients were tested for CASPR2 ab and found negative. The clinical findings, ancillary results and outcomes of the patients with CSF CASPR2 ab are shown in **Table 1**. The **Table 2** summarizes the baseline characteristics and immunological findings of CSF CASPR2 ab patients compared to NMT/MoS patients. Male predominance and median age were significantly higher in patients with CSF CASPR2 ab than in NMT/MoS patients (94% vs 67%, $p=0.03$ and 64.5 versus 51 years, $p<0.01$). Only one of the patients with CSF CASPR2 ab (6%) suffered from another autoimmune disorder (thyroiditis), whereas autoimmune co-morbidities were seen in 8/15 (53%) of the NMT/MoS patients ($p<0.01$; mostly myasthenia gravis: 7/8 patients). Only 3 CSF CASPR2 ab patients (17%) had a previous or concomitant history of cancer (prostate adenocarcinoma and chronic lymphoid leukemia in one patient and papillary thyroid cancer in two patients). Although a malignant thymoma had been found prior to or following the neurological disorder in 9/15 (60%) of the NMT/Mos patients, none of the patients with CSF CASPR2 ab had such a tumour ($p<0.01$). For a majority of the CSF CASPR2 ab patients (13/18, 72%), admission to the referring hospital occurred because of partial temporal seizures. In those cases, the other symptoms installed acutely over a few days to 1 week, simultaneously to epilepsy in 9 patients and subsequently in only 3

patients with a delay of a few months (median delay 6 months, range 5 – 18 months). In the remaining patients (5/18, 28%), admission was requested because of complaints of memory disorders, which had developed progressively over months in 4 of them and acutely in one patient. All CSF CASPR2 ab patients had symptoms of limbic structure impairment, including anterograde and episodic memory disorders (17/18, 94%), temporal lobe seizures (16/18, 89%), symptoms of frontal lobe dysfunction (10/18, 56%) or psychiatric symptoms (depressed mood or persecutory thoughts, 4/18, 22%). None of the patients presented with the acute confusion or behavioral disorders classically reported in other autoimmune limbic encephalitis. Extra-limbic symptoms were also observed in 12/18 (66%) patients, including cerebellar ataxia (6/18, 33%), sleep disorders (4/18, 22%) and peripheral neuropathy (3/18, 17%). Signs of neuromyotonia were present in only 2/18 (11%) patients.

Ancillary. Hyponatremia was observed in only one patient (6%). Anti-SOX-1 antibodies were detected in one patient, although no evidence of cancer was demonstrated. None of the other patients had additional antibodies against neuronal antigens. Brain magnetic resonance imaging (MRI) data were available for 15 patients. Brain MRI results remained normal in only one patient (7%). Twelve patients (80%) had temporo-limbic hyperintensities on T2-weighted images (**Figure 3**), which appeared subsequently in 3/12 patients (25%). Bilateral hippocampal atrophy was observed in 2 patients (13%) on the first imaging analysis. In one patient, temporo-mesial hyperintensities were observed in the first MRI and secondarily evolved into bilateral hippocampal atrophy. CSF cytochemical analysis data were available for 12 patients and were normal in only 3 cases (25%). Increased white blood cell counts were observed in 8 patients (67%, median: 4; range: 3 – 32 x 10^6 cells/mm^3), and elevated protein levels were observed in 1 patient (8%, 1.09 x 10^3 mg/l). Of note, brain MRI and CSF analysis were normal in all

NMT/MoS patients for whom data was available (11 and 9 patients, respectively). Interictal electroencephalograms displayed focal abnormalities in 4 patients (22%). Brain positron emission tomography scans (PET) were performed in 5 patients and were pathological in 4 (80%). PET findings were not reproducible from a patient to another: diffuse hypometabolism in 1 patient, temporo-mesial hypometabolism in 1 patient and fronto-temporal hypermetabolism in 2 patients. We were therefore unable to determine a pattern specifically associated with CASPR2 antibody-related encephalitis. Nonetheless, it has to be noted that, in contrast with anti-Lgi1 encephalitis patients (Navarro et al. 2016), increased metabolism in the basal ganglia was not observed in any of the 5 patients.

Treatments. Five patients (28%) were given oral steroids (1 mg/kg/day for several weeks) alone (1/4) or in combination with other treatments (3/4). Eleven (61%) patients were treated with intravenous immunoglobulins (IVIg; 0.4 g/kg/day for 3-5 days, 1 to 9 monthly courses), 8 patients (44%) with high dose corticosteroid boluses (0.5 - 1 g/day, 3 to 5 days, 1 to 6 monthly courses), 1 (6%) with plasmapheresis (6 monthly courses), 3 (17%) with intravenous cyclophosphamide (1 g/month, 2 to 8 courses) and 3 (17%) with rituximab (375 mg/m^2/wk, 4 courses). Three patients were given mycophenolate mofetil (1 g/day) on a long-term basis. Four patients (22%) were given 2 lines of treatment, and 2 patients (11%) needed three different lines of treatment.

Table 1. Clinical findings, brain MRI and evolution of patients with CSF CASPR2 ab.

Age, sex	Symptoms leading to admission	Other symptoms	Brain MRI hypersignal	Follow-up (mo)	Evolution	Relapse	mRS at onset	mRS at last visit	Residual symptoms
62, M	Partial temporal seizures	Anterograde and autobiographic memory impairment – acute, at the same time as epilepsy	Temporo mesial, bilateral	55	Improvement after 3 monthly courses of IGIV, 5 months after onset	1 year after onset – seizure and memory disorders	1	1	Defects in autobiographic memory
57, M	Partial temporal seizures	Autobiographic memory impairment, insomnia – acute, at the same time as epilepsy	Temporo mesial, left	114	With AED only, progressive improvement until stabilization 2 years after onset. 1 steroid bolus 6 years after onset: memory improvement	Neuromyotonia 8 years after onset, without worsening of encephalitis signs	2	2	Neuromyotonia
60, M	Anterograde and episodic memory impairment	Partial and tonic-clonic generalized seizures, hypersomnia, 2 weeks after the onset of memory impairment	Temporo mesial, bilateral	51	One course of IgIV, inefficient. Improvement with steroids and plasmapheresis 8 months after onset. After relapses: improvement with plasmapheresis and steroid increase.	2 relapses (15 and 28 months after onset): Increased seizures at steroid decrease.	2	1	Altered visuospatial memory
71, M	Partial temporal seizures	Autobiographic memory impairment, frontal syndrome, cerebellar syndrome, pseudo-bulbar syndrome – Acute, 18 months after onset of epilepsy	NA	46	Marked improvement after 6 courses of SMD that were started 23 months after disease onset. MMF was given as long-term immunosuppression.	No	4	1	Moderate anterograde amnesia, slightly unstable gait
60, M	Partial temporal seizures	Anterograde and autobiographic memory impairment and cerebellar ataxia – acute, at the same time as epilepsy	Normal	22	Spontaneous improvement 5 month after disease onset. One course of steroid boluses 6 months after onset. After relapse: no improvement despite steroid boluses, one course of IgIV and 8 courses of CPA	Increased seizures 7 months after onset, followed by worsening of cerebellar ataxia	2	3	Refractory temporal epilepsy, cerebellar ataxia
68, M	Partial temporal seizures	Anterograde memory impairment, frontal signs, cerebellar ataxia, mild neuromyotonia – acute, at the same time as epilepsy	Temporo mesial, left	11	Marked improvement on cognitive functions and gait after 6 courses of IgIV with steroid boluses started 1 month after onset	No	2	1	Mildly impaired executive functions at NPT
69, M	Partial temporal seizures	Anterograde memory impairment, frontal features, persecuted thoughts – Acute, 6 months after onset of epilepsy	Temporo mesial, right	25	Spontaneous improvement with AED and oral steroids, 2 years after onset.	No	2	0	None
66, M	Partial temporal and generalized seizures	Anterograde and autobiographic memory impairments, frontal features – Acute, 5 months after	Temporo mesial, bilateral	19	Improvement after 3 courses of IgIV and steroid boluses 9 months after onset. After relapse: Improvement with 6	Increased seizures and memory impairment 13	2	2	Slight autobiographic amnesia,

		onset of epilepsy			courses of IVIg and CPA	months after onset.			impaired executive functions
75, M	Partial temporal and generalized seizures	Anterograde memory impairment, frontal syndrome – acute, at the same time as epilepsy	Temporo mesial, right	3	One course of SMD bolus and IgIV	NA	3	NA	Follow-up < 6 months
69, M	Partial temporal seizures	Anterograde amnesia, inferior limb sensory neuropathy – acute, at the same time as epilepsy	NA	19	Dramatic improvement after 2 courses of IVIg, 17 months after onset	No	2	1	Slight memory and executive function impairment, neurogenic pain
61, M	Partial temporal seizures	Anterograde and autobiographic amnesia – acute, at the same time as epilepsy	Hippocampal atrophy	12	Greatly improved after 5 courses of IVIg, 10 months after onset. Treatment with RTX due to the persistence of detectable CSF CASPR2 ab	No	1	1	Complaints of memory troubles and difficulty to concentrate
63, M	Partial temporal seizures	Anterograde amnesia, anxiety – acute, at the same time as epilepsy	Temporo mesial, bilateral	59	Improvement with steroids 3 months after onset. After relapses: partial improvement after IgIV and steroid increase. Inefficiency of MMF. Return to baseline after Rituximab therapy	2 relapses (3 and 13 months from onset): Increased seizures at steroid decrease	2	0	None
61, M	Partial temporal and generalized tonic-clonic seizures	Slight frontal syndrome – acute, at the same time as epilepsy	Temporo mesial, left	78	Spontaneous improvement in seizure frequency 2 years after onset.	No	1	1	Daily partial temporal seizures
62, F	Partial temporal seizures	Anterograde and autobiographic amnesia, frontal signs, hemihypesthesia, insomnia, depressed mood – acute, at the same time as epilepsy	Hippocampal atrophy	29	Improvement with IVIg at 18 months from onset	No	2	1	Slight anterograde amnesia and executive impairment at NPT, insomnia
53, M	Progressive (several months) autobiographic amnesia, axonal sensory-motor neuropathy with neuralgic pain	Frontal features, cerebellar ataxia, insomnia, depressed mood	NA	25	Greatly improved after 6 steroid boluses, begun at 25 months from onset	No	3	1	Neuropathic pain, depressed mood
66, M	Progressive amnesia, cerebellar ataxia, sensory neuropathy	Partial temporal seizures, frontal features – in parallel to progressive amnesia, over several months	Temporo mesial, bilateral	5	Partial improvement after 4 courses of IVIg started 1 month after onset	NA	3	NA	Follow-up < 6 months
75, M	Progressive	None	Temporo	39	Stabilisation after 9 courses of IVIg 1 year	No	4	4	Severe

	amnesia, aphasia, cerebellar ataxia – installation over several months		mesial, bilateral		after onset. Switch to CPA afterwards, no improvement				anterograde amnesia, ataxia, aphasia (4)
66, M	Progressive anterograde amnesia	Partial temporal seizures, tetrapyramidal signs, frontal features – in parallel to amnesia, over several months	Temporo mesial, bilateral and later hippocampal atrophy	92	9 steroid boluses started 50 months after onset. Afterwards, progressive improvement. MMF as long-term immunosuppressive therapy.	No	4	1	Slight anterograde amnesia (1)

	CSF CASPR2 Ab	NMT/MoS	Statistical value
Number of patients	18	15	
Males/females (ratio)	17/1 (17)	9/6 (1.5)	p = 0.03
Median age (range)	64.5 (53 – 75)	51 (1 month – 75y)	p < 0.01
Median Serum titres (range)	1/15,360 (10,240 – 81,920)	1/800 (20 – 5120)	p < 0.0001
Median CSF titres (range)	1,280 (10,240)	0 (0 – 0)	p < 0.0001
Detected IgG4 CASPR2 Ab			
Serum	11/12 (92%)	5/12 (42%)	p = 0,03
CSF	17/17 (100%)	0/15 (0%)	p < 0.0001
Detected IgG1 CASPR2 Ab			
Serum	12/12 (100%)	10/12 (83%)	Not significant
CSF	10/17 (59%)	0/15 (0%)	p < 0.005
Recognition of discoidin and laminin G1	18/18 (100%)	11/11 (100%)	Not significant
Additional epitopes	10/18 (56%)	8/11 (73%)	Not significant
Malignant Thymoma	0/18 (0%)	9/15 (60%)	p < 0.01
Other cancers	3/18 (17%)	0/15 (0%)	Not significant
Other autoimmune disturbances	1/18 (6%)	8/15 (53%)	p < 0.005

Table 2. Baseline characteristics and immunological findings of patients with CSF CASPR2 ab compared to NMT/MoS patients.

Follow-up and outcomes. The median follow-up time was 27.4 months (IQR 19 – 51 months; range 3 – 114 months). All patients survived. Follow-up was performed for at least 6 months in 16 out of 18 patients (89%). Fifteen of them (94%) progressively improved, after immunomodulatory treatment (13 patients) or spontaneously (2 patients) (**Table1**). One patient remained stable with ataxia and severe cognitive defects. Six patients (38%) had one or two relapses. Five of them had relapses presenting as an increase in seizure frequency. In 2 patients, epileptic relapses occurred when steroid medications were decreased and were easily controlled by increasing steroid dosages. The relapse of one patient consisted in the development of neuromyotonia 8 years after the onset of the disease, without concomitant worsening of the encephalitic symptoms. The median modified Rankin score for all patients was 2 at the onset (IQR 2 – 3; range, 1 – 4) and 1 at the end of follow-up (IQR, 1 – 2; range, 0 – 4). At the end of follow-up 4 patients (25%) still had symptoms that significantly altered their quality of life (mRS≥2), including neuromyotonia (1 patient), cerebellar ataxia (2 patients) and amnesia (2 patients). Two out of 16 patients (13%) recovered completely. Brain MRI follow-up data were available for 9 patients. The MRI results remained stable in 6 cases (67%), while temporo-mesial hyperintensities decreased partially or completely in 2 patients (22%) and evolved from unilateral to bilateral in one patient (11%). One patient (11%) secondarily developed bilateral hippocampal atrophy.

CASPR2 antibody characteristics. As reported before(Lancaster et al. 2011), CSF immunohistochemical studies revealed in all patients a similar staining pattern with strong fixation of the granular and molecular layers of the cerebellar cortex and hippocampus along with a diffuse staining of the molecular layer of the dentate gyrus (**Figure 1**). Median CSF CASPR2 ab end point dilution was 1/1280 (range, 1/80 – 1/10,240). All the serum samples from the patients were also positive for CASPR2 ab.

Median serum CASPR2 ab end point dilution were significantly higher in patients with CSF CASPR ab than in NMT/MoS patients (1/15,360 versus 1/800, $p<0.0001$). CSF and serum albumin levels were available for two patients, allowing for the assessment of intrathecal synthesis. In both of the patients, the QIg values (0.03 and 0.06) were larger than the QLim values (0.004 and 0.011). The CASPR2 antibody indices were 7.4 and 5.6, suggesting intrathecal synthesis of CASPR2 ab in both patients. In all patients, serum and CSF CASPR2 ab were directed against the discoidin and laminin G1 domains of CASPR2, as illustrated in **Figure 2A**. IgG isotypes were determined in 17 patients (**Figure 2B**). Importantly, IgG4 antibodies were detected in the CSF of all patients (17/17) along with IgG1 antibodies in 10/17 patients (59%). Interestingly, serum IgG4 CASPR2 ab were found in 11/12 (92%) of the CSF CASPR2 ab patients and in only 5/12 (42%) of the NMT/MoS patients ($p=0.03$). We observed 3 patients with CSF CASPR2 ab that were of the IgG4, but not IgG1 isotype, which targeted the discoidin and laminin G1 domains only. All three of these patients had anterograde and episodic amnesia and temporal seizures. One patient also complained of persecutory thoughts, and another patient exhibited hypersomnia. Notably, all 3 patients with hippocampal atrophy on brain MRI had CSF CASPR2 ab of both the IgG4 and IgG1 isotypes.

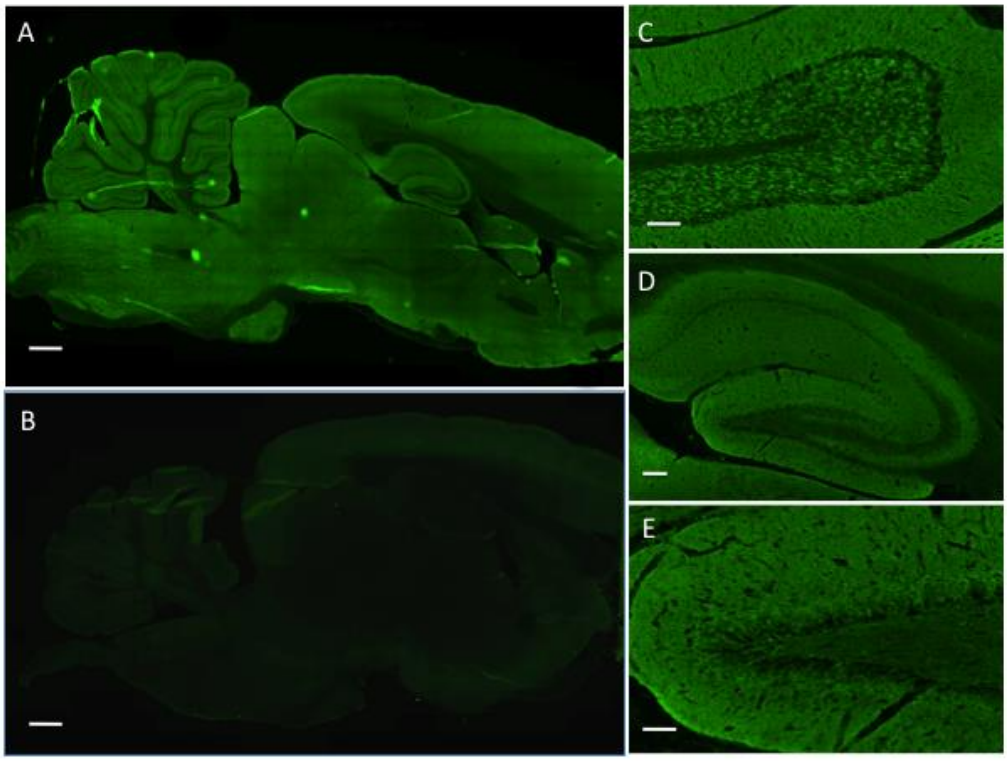

Figure 1: **Reactivity of patient CSF CASPR2 antibodies with rat brain.**
(A) Sagittal section of rat brain immunostained with a patient's CSF (dilution 1:10). There was diffuse staining of the neuropil that was not observed when rat brain sections were incubated with control CSF. (B) Immunoreactivity was particularly strong in the molecular and the granular cell layers of the cerebellum (in C) and the hippocampus (in D); however, there was diffuse staining of the molecular layer of the dentate gyrus (in E). Scale bars in A and B=1000 μm, in D=200 μm, and in C and E=100 μm.

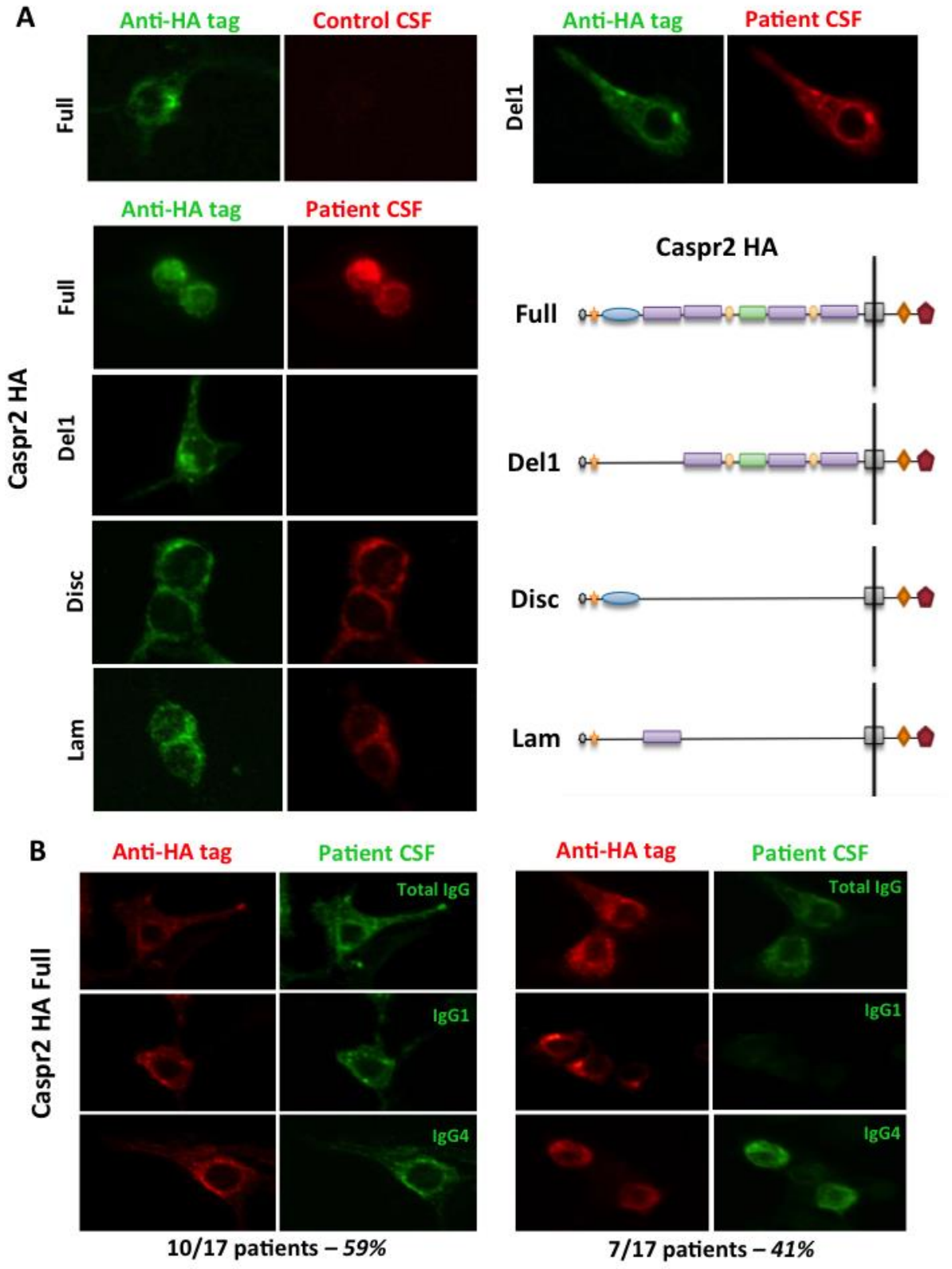

Figure 2: CASPR2 autoantibody characterization.

(A) Determination of the domains targeted by CASPR2 autoantibodies derived from the CSF of the patients (dilution 1:10). HEK cells were transfected to express the HA-tagged full-length CASPR2 protein or domain deletion constructs. The CASPR2 Del1 construct, lacking the Discoidin-Laminin domains was not recognized by 8/18 patients as illustrated here for patients 7 and 16, whereas constructs only including the discoidin domain, laminin G1 domain or both domains (not shown) were recognized by all CSF samples.
(B) Determination of CASPR2 autoantibody IgG isotypes in the CSF of the patients (dilution 1:10). HEK cells were transfected to express the HA-tagged full-length CASPR2 protein. The IgG isotype of patient autoantibodies bound to the CASPR2 protein was then characterized using secondary antibodies directed against IgG1 or IgG4 antibodies. All patients had IgG4 antibodies but IgG1 antibodies were detected in only 10/17 patients.

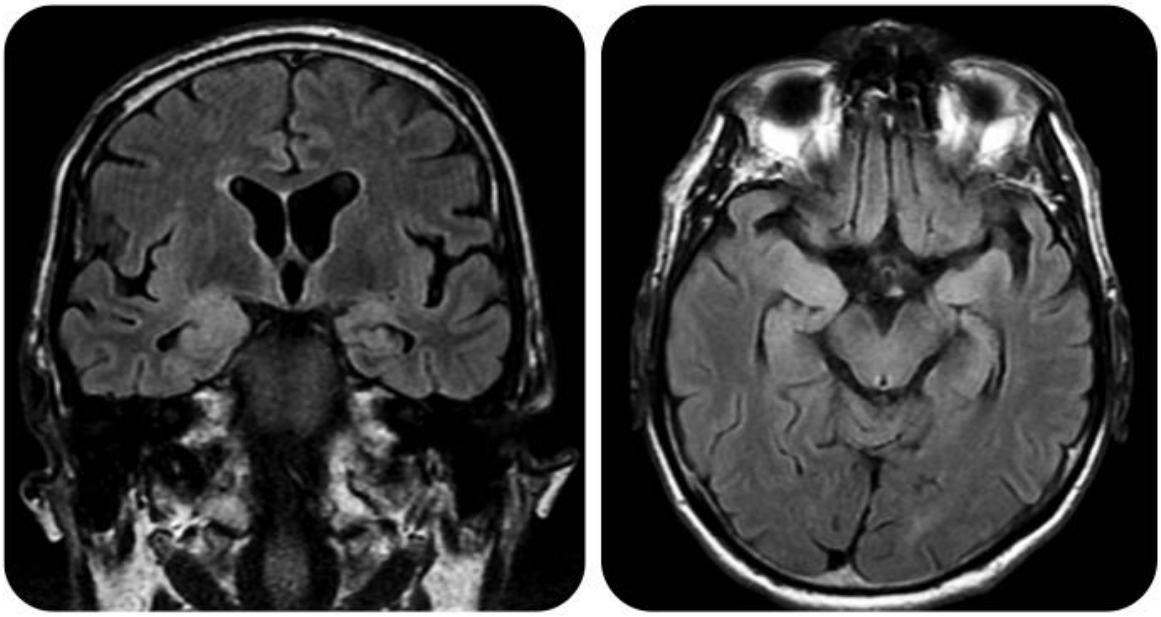

Figure 3: Brain magnetic resonance imaging of patient 7.
Fluid-attenuated inversion recovery sequences, left: axial plane, and right: coronal plane.
In the T2-weighted MRI image, note the asymmetrical hyperintensities involving both temporo-mesial regions.

DISCUSSION

Clinical patterns. It has been previously reported that patients with CASPR2 ab present with symptoms of neuromyotonia, Morvan's syndrome and various patterns of autoimmune encephalitis(Lancaster et al. 2011; Becker et al. 2012; Laurencin et al. 2015; Sunwoo et al. 2015). However, our study indicates that the presence of CASPR2 ab in the CSF is associated with a homogeneous clinical pattern of autoimmune encephalitis with prevalent limbic involvement and seizures. As reported before(Irani et al. 2010; Lancaster et al. 2011), we observed an overrepresentation of male patients in our cohort, which was significantly higher than in NMT/MoS patients. Epilepsy was the main symptom that led the patients to seek medical attention. Neither confusion nor behavioral disorders were observed in any of the patients. All patients had anterograde or episodic memory disorders and/or temporal seizures that suggest a constant involvement of the hippocampus. Brain MRI images were in accordance with this result as the majority of the patients displayed temporo-mesial hyperintensities or hippocampal atrophy on T2-weighted images (**figure 3**). Nonetheless, extra-limbic symptoms were observed as well, the most frequent being cerebellar ataxia, in accordance with previous results(Becker et al. 2012). Neuromyotonia was rare in CSF CASPR2 ab patients. Relapses were observed in 6 patients, most often in the form of an increase in seizure activity. In some cases, this increased seizure activity was steroid-dependent and steroid-responsive, indicating the possible direct responsibility of anti-CASPR2 autoimmunity for the epileptogenic process. By contrast with NMT/MoS patients, none of the CSF CASPR2 ab patients had a malignant thymoma.

Our data suggest that CASPR2 ab when detected in the CSF associate with a subtype of autoimmune encephalitis that is mostly non-paraneoplastic. By comparison, NMT/MoS patients do not have detectable CSF CASPR2 ab and are frequently affected by a

malignant thymoma. The absence of detectable CASPR2 ab in the CSF of NMT/MoS could indicate an autoimmune reaction developed strictly outside of the CNS. Alternatively, as serum titres were much lower in NMT/MoS patients, the absence of CSF CASPR2 ab in those patients might reflect extremely low titres of CASPR2 ab in the CSF. Therefore, our data suggest that the observed differences in clinical presentation between the 2 groups are underlined by variations of the autoantibodies production within the intrathecal compartment.

Epitope specificities. Interestingly, the CSF CASPR2 ab from all patients targeted the discoidin and laminin G1 domains of CASPR2, suggesting that recognition of these domains is involved in the genesis of symptoms. To our knowledge, the precise roles of the discoidin and laminin G1 domains have not yet been clearly determined.

IgG subtypes. Importantly, IgG4 CASPR2 ab were detected in the CSF of all patients. IgG4 antibodies cannot bind to complement or form immune complexes and have a low affinity for Fc receptors(Bindon et al. 1988; Bruhns et al. 2009). Moreover, IgG4 antibodies have the ability to exchange Fab arms, and a fraction of them are therefore bispecific, suggesting that some of the CASPR2 ab of our patients may have bispecificity for the laminin G1 and discoidin domains(Schuurman et al. 1999; van der Neut Kolfschoten et al. 2007). Interestingly, Fab fragments of anti-Musk IgG4 autoantibodies from myasthenia gravis patients have been shown to be pathogenic *in vitro*, suggesting the involvement of mechanisms other than the classical cross-linking and internalization that was observed with other autoantibodies directed against synaptic proteins(Huijbers et al. 2013). It remains to assess whether IgG4 CASPR2 ab are bispecific for the discoidin and laminin G1 domain and whether they are able to cross-link CASPR2 and induce its internalization or alter its function by other mechanisms. Besides, IgG1 autoantibodies were found in all the patients with hippocampal atrophy,

implying the possible involvement of complement or antibody-dependent cell death. This view is supported by one biopsied case of CASPR2 antibody encephalitis in which immunoglobulin and complement depositions were reported in neurons along with cell degeneration in the hippocampus(Körtvelyessy et al. 2015). Therefore, distinct pathological processes can occur during the course of CASPR2 antibody encephalitis, ranging from functional alterations involving IgG4 autoantibodies to complement-mediated cell toxicity induced by IgG1 autoantibodies.

In conclusion, CASPR2 ab are found in the CSF only in patients with CASPR2 antibody-associated autoimmune encephalitis whereas in NMT/MoS patients CASPR2 ab are detected only in the serum. CSF CASPR2 antibody-associated encephalitis is observed predominantly in males and is characterized by prominent limbic symptoms and temporal lobe epilepsy. Recognition of the discoidin and laminin G1 domains by the autoantibodies is constant and may be important in the genesis of limbic symptoms. Additionally, CASPR2 ab of the IgG4 isotype may play a fundamental role in the pathogenic processes through a functional effect on CASPR2, although complement-mediated cell death induced by IgG1 autoantibodies may also occur in some patients. These hypotheses must be further supported by future functional studies focused on antibody specificities.

Commentary.

In this first study, we assessed the clinical features and outcomes of 18 patients with CASPR2-Ab autoimmune encephalitis. The diagnosis of autoimmune limbic encephalitis was based on the presence of at least one symptom of limbic dysfunction (temporal lobe seizures, or anterograde amnesia). We considered Morvan syndrome as an entity distinct from limbic encephalitis, based on the historical clinical description by Roger et al, 1953, and patients considered as having Morvan syndrome were excluded from the analysis (Roger, Alliez, and Roger 1953; Irani et al. 2012). The most relevant features of our limbic encephalitis patients are the affected population (almost all were males aged from 50 to 75 years), the frequent presence of non-limbic symptoms, and the usually good response to immunotherapy. Most cases had an acute initial presentation, with amnesia and seizures, but in 20% the first symptom was memory impairment installed progressively over months. Considering the non-limbic symptoms, ataxia is particularly frequent (up to one-third of the patients), and 2 patients had neuromyotonia in addition to the limbic encephalitis symptoms. Most patients improved after immunotherapy, but 25% were left with moderate to severe neurological symptoms, including temporal lobe epilepsy, cerebellar ataxia, and memory impairment. The study is limited by the relatively small number of patients and the fact that data collection was retrospective. Nonetheless, our findings have been confirmed by other studies, which noted in addition that many CASPR2-Abs patients lose weight, and that some patients had hyperkinetic movement disorders (van Sonderen, Ariño, et al. 2016; C. G. Bien et al. 2017). However, the study by Van Sonderen and colleagues had a different approach since they did not make the distinction between limbic encephalitis and other syndromes, considering CASPR2-Abs clinical spectrum as a whole. Consequently, they claimed that the patient did not conform to specific syndromes, but instead had a set of core symptoms forming random combinations (van Sonderen, Ariño, et al. 2017). In favor of this concept, we found that

CASPR2-Abs recognized the same epitopes on the distal discoidin and laminin G1 domains of CASPR2, regardless of the clinical presentation. More in favor of the syndromic classification however, is our finding that the patients who had a limbic encephalitis syndrome, with or without additional symptoms, had markedly higher serum levels of CASPR2-Ab and their CSF tested positive for CASPR2-Abs, in contrast with those with exclusively non-limbic syndromes (Morvan or NMT), who had lower serum titers and CASPR2-Ab negative CSF. Also, the frequence of the IgG4 isotype of CASPR2-Abs among patients differed in limbic encephalitis patients compared to Morvan syndrome or neuromyotonia patients. While in serum the IgG1 isotype was found in most patients regardless of the clinical phenotype, the IgG4 isotype was found in 92% of the limbic encephalitis patients compared to only 42% of the neuromyotonia and Morvan syndrome patients. In addition, CASPR2-Abs found in the CSF of limbic encephalitis patients were predominantly of the IgG4 isotype. Whether these differences in IgG isotypes play a role in pathophysiology and the determination of the phenotype, is unclear. These findings may suggest that neurological symptoms do not form random combinations, and that antibody titres, isotype, and site of synthesis correlate with the neurological phenotype.

SECOND STUDY

AUTOIMMUNE EPISODIC ATAXIA IN CASPR2-ABS PATIENTS

Title: Autoimmune episodic ataxia in patients with anti-CASPR2 antibody-associated encephalitis.

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ABSTRACT

Objectives: To report paroxysmal episodes of cerebellar ataxia in a patient with anti-contactin-associated protein-like2 (CASPR2) antibody-related autoimmune encephalitis and to search for similar paroxysmal ataxia in a cohort of anti-CASPR2 antibody-associated autoimmune encephalitis patients.

Methods: We report a patient with paroxysmal episodes of cerebellar ataxia observed during autoimmune encephalitis with anti-CASPR2 antibodies. In addition, clinical analysis was performed in a retrospective cohort of 37 patients with anti-CASPR2 antibodies to search for transient episodes of ataxia. Paroxysmal symptoms were further specified from the referral physicians, the patients or their relatives.

Results: A 61 year-old male with limbic encephalitis and anti-CASPR2 antibodies developed stereotyped paroxysmal episodes of cerebellar ataxia, including gait imbalance, dysarthria and dysmetria, 1 month after the onset of the encephalitis. The ataxic episodes were specifically triggered by orthostatism and emotions. Both limbic symptoms and transient ataxic episodes resolved after treatment with steroids and intravenous cyclophosphamide. Among 37 other patients with anti-CASPR2 antibodies, we identified 5 additional cases with similar paroxysmal ataxic episodes that included gait imbalance (5 cases), slurred speech (3 cases), limb dysmetria (3 cases), and nystagmus (1 case). All had concomitant limbic encephalitis. Paroxysmal ataxia was not observed in patients with neuromyotonia or Morvan's syndrome. Triggering factors (orthostatism or anger) were reported in 4 patients. Episodes resolved with immunomodulatory treatments in 4 patients and spontaneously in 1 case.

Conclusion: Paroxysmal cerebellar ataxia must be added to the spectrum of the anti-CASPR2 antibody syndrome.

INTRODUCTION

Episodic ataxias (EA) are a group of hereditary channelopathies whose common feature is the occurrence of paroxysmal episodes of cerebellar ataxia (Tomlinson et al. 2009). Ataxic episodes usually last a few minutes to a few days and can be triggered by emotions, abrupt movements, exercise, or fever. Depending on which ion channel gene is mutated, additional symptoms, such as neuromyotonia or epilepsy, can occur (D'Adamo et al. 2015). Conversely, paroxysmal symptoms are rare in patients with anti-neuronal antibody-associated neurological disorders, and episodic ataxias have not yet been reported in such cases. In this study, we report a patient with paroxysmal episodes of ataxia developed during autoimmune encephalitis with anti-CASPR2 antibodies. In order to assess the relevance of our case, we retrospectively searched for similar episodes of transient ataxia in a cohort of patients with anti-CASPR2 antibodies. Anti-CASPR2 antibody-related disorders encompass a wide range of neurological autoimmune syndromes including autoimmune encephalitis, neuromyotonia and Morvan's syndrome. A recent publication by Van Sonderen et al showed that up to 77% of the patients with such antibodies had at least 3 cumulated core neurological symptoms, including encephalic signs, cerebellar symptoms, peripheral nerve hyperexcitability, dysautonomia, neuropathic pain, insomnia and weight loss(van Sonderen, Ariño, et al. 2016). Our study may contribute to further delineate anti-CASPR2 antibody-related clinical presentation.

METHODS

We report a patient with autoimmune encephalitis, anti-CASPR2 antibodies and paroxysmal cerebellar ataxia. Anti-CASPR2 antibodies were screened in serum and cerebrospinal fluid (CSF) as previously described (Joubert et al. 2016). Positivity of both an immunohistofluorescent assay on rat brain slices and a cell-based binding assay with HEK-

293 transfected cells were needed to confirm the presence of anti-CASPR2 antibodies. A signed Patient Consent-to-Disclose Form has been obtained from the patient for a video recording one of the episodes.

We also studied the clinical files of 37 patients with anti-CASPR2 antibodies, detected in their CSF or sera at the *Centre de Référence National pour les Syndromes Neurologiques Paranéoplasiques* (Lyon, France) between March 2009 and August 2016, to search for similar transient cerebellar symptoms. Thirty-three of those patients have been previously reported (Joubert et al. 2016). Written informed consent was obtained from all patients with approval of the institutional review board of the Hospices Civils de Lyon. We selected all patients who had been reported by their referral physicians to have symptoms that were both transient and suggestive of cerebellar impairment, i.e. gait imbalance, slurred speech or limb dysmetria. Further information was collected by telephone from the referral physicians, and, when possible, from the patients themselves or their relatives.

RESULTS

Index case: A 61 year-old man was hospitalized for evaluation after a tonic-clonic generalized seizure. He was still active as a corporate executive and his previous medical history included high blood pressure, diabetes, myocardial infarction, and a smoking habit. No prodromal or post-ictal symptom was reported, but the patient reported slight memory impairment, difficulties to concentrate at work, unusual emotiveness and anxiety, over a few days before the seizure. The patient was treated with levetiracetam and clobazam. However, the cognitive symptoms persisted and several partial temporal lobe seizures occurred. One month after the first seizure, the patient began to experience repeated episodes of slurred speech, gait ataxia, and slight dysmetria of the limbs. These events occurred 3 to 4 times a day, were often triggered by emotion and orthostatism, and lasted less than 1 minute. No cerebellar symptom

was observed between the episodes. Levetiracetam was switched to lacosamide, without any improvement of the symptoms.

Brain MRI was normal. EEG found an asymmetric temporal lobes slowing. CSF analysis found 16 white blood cells (WBC)/mm^3, a protein level of 65 mg/dL, and no oligoclonal band. Anti-CASPR2 antibodies were detected (end point dilution using cell-binding based assay, 1/40,960 in serum and 1/5,120 in CSF). Brain single-photon emission computerized tomography found bilateral fronto-temporal hypoperfusion, with normal perfusion of basal ganglia and cerebellum. Full-body computerized tomography was normal. Sequencing of the genes involved in EA types 1 and 2, *KCNA1* and *CACNA1A*, found that the patient carried a rare polymorphism in intron 39 of *CACNA1A* (c5843-14G>A) (Browne et al. 1994; Terwindt et al. 1998). *KCNA1* was normal. The patient was treated with 3 daily injections of 1g methylprednisolone followed by 6 monthly pulses of 1g cyclophosphamide. The frequency of the ataxia episodes decreased immediately; they had totally stopped 5 days after the end of the methylprednisolone pulses. The patient recovered all his cognitive abilities 2 weeks after initiation of the treatment and had his last seizure 3 months after first visit. At month 7 of follow-up, the patient remained asymptomatic and seizure-free. At the peak of his disease, the patient had presented with only 2 core symptoms as defined by Van Sonderen et al (encephalic signs and cerebellar ataxia)(van Sonderen, Ariño, et al. 2016).

Cohort study.

Among the cohort of 37 other patients with anti-CASPR2 antibodies (20 with encephalitis and 17 with neuromyotonia or Morvan's syndrome), we retrospectively identified 5 patients with transient symptoms suggestive of cerebellar impairment. All the patients were male, with a median age of 60 years (range, 57 – 69). Median follow-up (range) was 45.6 months (19.3 – 113.6). One patient had a previous history of surgically treated thyroid carcinoma. All these 5

patients had anti-CASPR2 antibodies in both serum and CSF and had a presentation of autoimmune encephalitis with prominent seizures and amnesia, as we previously reported (Joubert et al. 2016). Three patients also developed mild permanent cerebellar symptoms, concomitantly to (2 cases) or after (1 case) the onset of paroxysmal ataxia.

None of them exhibited neuromyotonia or Morvan's syndrome. Two of the 5 patients had ≥3 core symptoms as defined in the study by Van Sonderen et al (van Sonderen, Ariño, et al. 2016). The transient episodes are described in the Table. They were stereotyped and included, according to the cases, gait imbalance (5/5, 100%), slurred speech (3/5, 60%), limb dysmetria (3/5, 60%), and nystagmus (1/5, 20%). Two patients (40%) reported concomitant sensations of neck stiffness. Myoclonic jerks were not observed in any of the patients. Interestingly, the ataxia episodes preceded by 7 months the full development of the encephalitis in 1 patient. The apparition of the ataxia was not subsequent to a change of antiepileptic medication in any of the patients. The attacks were reported to last from a few minutes to 2 hours, according to the cases, with triggering factors identified in 4 patients (80%; orthostatism in 4 patients, anger in 1 patient). Ataxia episodes occurred during a mean period of 4 months (range, 0.5 – 32.5) and stopped after initiation of an immunomodulatory treatment in 4 patients (steroids alone, steroids and plasmapheresis, steroids and intravenous immunoglobulin, or rituximab) and spontaneously in one case. Interictal, milder cerebellar symptoms were observed in 3/5 cases (60%). Interictal brain MRI was unremarkable in all patients; magnetic resonance spectroscopy was not performed during the episodes. We did not perform genetic studies in these 5 patients.

Table. Characteristics of the ataxia episodes in patients with autoimmune encephalitis and anti-CASPR2 antibodies. The first line refers to the index case.

Age (yrs), sex	Follow-up (mo)	Symptoms during the episodes	Triggering factors	Impact on daily activities	Duration of the episodes	Frequency at peak	Permanent cerebellar symptoms also present	Onset in relation to the onset of the limbic symptoms	Period during which paroxysmal ataxia occurred	Anti-CASPR2 ab titres		Treatments before resolution of the episodes
										Serum	CSF	
61, M	7	Gait imbalance, slurred speech, limb dysmetria	Anxiety, orthostatism	No	< 1 min	3 - 4 / day	No	1 month later	3 weeks	1/40960	1/1520	Methylprednisolone, cyclophosphamide
62, M	114	Gait imbalance, slurred speech	None	No	A few minutes	2 episodes with an interval of 2 weeks	No	10 months later	2 weeks	NA	1/320	None
57, M	51	Gait imbalance	orthostatism	No	A few minutes to 2 hours	3 - 5 / week	No	4 months later	4 months	1/10240	1/640	Methylprednisolone, plasmapheresis
60, M	46	Gait imbalance, slurred speech, limb dysmetria, neck stiffness	orthostatism	Yes	A few minutes	2 - 3 / day	Yes (Gait imbalance, inferior limb dysmetria)	7 months before	33 months	1/10240	1/10240	Methylprednisolone
60, M	22	Gait imbalance, slurred speech, limb dysmetria, nystagmus, neck stiffness	Orthostatism, anger	Yes	A few minutes	6 - 7 / day	Yes (slight imbalance)	Concomitant	13 months	1/10240	1/10240	Rituximab
69, M	19	Gait imbalance, limb dysmetria	orthostatism	No	< 1 min	< 5 / week	Yes (slight imbalance)	17 months later	2 months	1/10240	1/10240	Prednisolone, intravenous immunoglobulin

Abbreviations: M, male; CSF, cerebrospinal fluid; ab, antibodies, NA, not available.

DISCUSSION

In this study, we report stereotyped episodes of paroxysmal cerebellar ataxia in a patient with anti-CASPR2 antibody-related autoimmune encephalitis. We also retrospectively identified similar paroxysmal symptoms in 5 out of 20 other patients with autoimmune encephalitis and anti-CASPR2 antibodies, suggesting that episodic cerebellar ataxia could be observed in up to 25% of such patients.

As they developed during the course of encephalitis and resolved after immunomodulating therapy in most of the patients, we can speculate an immune origin of these paroxysmal ataxias. Moreover, the association of gait imbalance, slurred speech, and limb dysmetria during these episodes was highly suggestive of cerebellar impairment, although due to the retrospective collection of the data, we cannot completely exclude that the alleged cerebellar symptoms were actually of another nature (e.g. orthostatic hypotension, seizures, or peri-ictal autonomic manifestations).

Paroxysmal neurological symptoms have already been reported in autoimmune encephalitis. For instance, facio-brachial dystonic seizures are caused by the activation of the motor cortex in patients with anti-Leucin-rich glioma1 (Lgi1) antibody-associated encephalitis (Navarro et al. 2016). Interestingly, Lgi1 is a secreted protein that complexes with CASPR2 and the potassium channel $K_V1.1$ (Browne et al. 1994; Lai et al. 2010). More recently, orthostatic myoclonus has been recently observed in a patient with anti-CASPR2 antibodies(Gövert et al. 2016). Although permanent ataxia without remissions is a well-described feature of anti-CASPR2 antibody-associated encephalitis, to our knowledge, episodic ataxia has never been reported before in autoimmune encephalitis patients, including for instance patients with anti-Lgi1 encephalitis (Joubert et al. 2016; Becker et al. 2012; Balint et al. 2013; van Sonderen, Ariño, et al. 2016).

Anti-CASPR2 antibodies associate with various neurological disorders, including limbic encephalitis, neuromyotonia and Morvan's syndrome (Irani et al. 2012; Lancaster et al. 2011; van Sonderen, Ariño, et al. 2016; Joubert et al. 2016; C. G. Bien et al. 2017). In a recent published cohort of 38 anti-CASPR2 antibody-positive patients, 77% of the cases had ≥3 core symptoms (encephalic signs, cerebellar symptoms, peripheral nerve hyperexcitability, dysautonomia, neuropathic pain, insomnia and weight loss)(van Sonderen, Ariño, et al. 2016). In our previously published cohort of 33 patients with anti-CASPR2 antibodies, only 16/33 (48%) of the patients presented with ≥3 of those core symptoms (Joubert et al. 2016). However, patients with limbic encephalitis were overrepresented and we found a greater number of patients with ≥3 core symptoms in the group of patients diagnosed with neuromyotonia or Morvan's syndrome (13/15, 87%), than in the group of patients diagnosed with limbic encephalitis (3/18, 17%). Only 2/6 (33%) of our patients with paroxysmal ataxia had ≥3 core symptoms, which reflects that this paroxysmal syndrome is mostly associated with limbic encephalitis rather than neuromyotonia or Morvan's syndrome.

The resemblance of the patients' paroxysmal ataxia with manifestations observed in hereditary channelopathies such as EA is striking. Interestingly, neuromyotonia can be observed in EA type 1 and anti-CASPR2 antibodies patients, suggesting similar ion channel dysfunctions in both diseases. EA type 1 is due to mutations of *KCNA1*, a gene coding for the voltage-gated potassium channel $K_V1.1$ (Browne et al. 1994; Lancaster et al. 2011). The clustering of $K_V1.1$ at the nodes of Ranvier depends on CASPR2 and electrophysiological experiments have suggested an impairment of voltage-gated potassium channels in autoimmune neuromyotonia, implying that anti-CASPR2 antibodies might indirectly alter the functions of $K_V1.1$ at the nodes of Ranvier (Horresh et al. 2008; Sinha et al. 1991). However, previous studies have failed to demonstrate a role of CASPR2 in $K_V1.1$ clustering at the synaptic level and the exact role of anti-CASPR2 antibodies in the CNS is unknown (Sinha et

al. 1991; Ogawa et al. 2008). Nevertheless, our observation supports the hypothesis of immune-mediated, ion channel dysfunction in anti-CASPR2 antibodies syndromes. The polymorphism of *CACNA1A* in the index patient is interesting because this gene codes for the $Ca_V2.1$ subunit of the P/Q type voltage-gated calcium channel that is mutated in EA type 2 patients (Terwindt et al. 1998). We have no data about the significance of this polymorphism, whose overall frequency in the general population is estimated at less than 1/1000. However, we can hypothesize that it may provoke a partial impairment of $Ca_V2.1$ (Terwindt et al. 1998). We can thus speculate that in the case this polymorphism of *CACNA1A* may have lead to the failure of compensatory mechanisms dependent on $Ca_V2.1$ and necessary to counterbalance the effects of anti-CASPR2 antibodies. Therefore, this polymorphism may have favoured the development of episodic cerebellar symptoms in the patient.

Overall, our findings suggest that transient cerebellar ataxia should be added to the spectrum of anti-CASPR2 antibody-related symptoms. Such paroxysmal symptoms are similar to the symptoms of hereditary channelopathies, suggesting that ion channel dysfunction is involved in the pathogenesis of anti-CASPR2 antibody syndromes.

Commentary.

In this study, we report the first cases of autoimmune episodic ataxia, a novel clinical sign associated with CASPR2-Abs autoimmune encephalitis. Among a cohort of 37 patients with CASPR2-Abs, we identified 6 patients who presented with paroxysmal episodes of cerebellar ataxia. All patients involved also developed limbic symptoms at some point, suggesting this new symptom is strongly linked to autoimmune limbic encephalitis. All patients had axial symptoms (gait instability, slurred speech) and four out of six patients had also limb ataxia. The bouts of ataxia were usually brief (a few minutes long) and could be triggered by external factors such as orthostatism or emotional stress. Episodic ataxia resolved in all patients, in most cases after treatment with steroids and other immunotherapies, but half of the patients developed permanent cerebellar ataxia. Orthostatic myoclonus, a similarly transient, stimulus-dependent neurological symptom, had been previously reported in one CASPR2-Abs patient but ours is the first reported series of immune-mediated episodic ataxia (Gövert et al. 2016). Interestingly, autoimmune episodic ataxia features are reminiscent of hereditary episodic ataxia, which is due to mutations in the genes coding for voltage-gated ion channels, including the VGKC subunit Kv1.1, which is known to interact closely with CASPR2 (Baloh 2012; Inda, DeFelipe, and Muñoz 2006). Remarkably, we found 2 patients with CASPR2-abs autoimmune encephalitis and episodic ataxia who had rare polymorphisms in either Kv1.1 or Ca2.1 coding genes, which when mutated can cause hereditary episodic ataxia type 1 and 2, respectively (Baloh 2012). Many mutations have been identified in episodic ataxia type 1 and 2 patients, with diverse effects on ion channel function, assembly, or membrane targeting(Jen and Wan 2018). In the case of our CASPR2-Abs patients with autoimmune episodic ataxia, the impact the polymorphisms have on ion channel function is unknown. It can be speculated that, by modulating how the central nervous system compensates the effects of CASPR2-Abs,

they represent predisposing factors for the development of ataxia in patients with CASPR2-Ab.

THIRD STUDY

CLINICAL PATTERNS DISTRIBUTION IN CASPR2-ABS PATIENTS

Title: Clinical phenotypes in CASPR2-antibody diseases correlate with distinct HLA associations and immunological features

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Abstract

Objective: Antibodies against contactin-associated protein-like 2 (CASPR2-Ab) are associated with acquired neuromyotonia (NMT), limbic encephalitis (LE) and Morvan syndrome (MoS), but recent studies suggest a wider and overlapping spectrum. Herein, we investigated the distribution of symptoms in CASPR2-Ab patients.

Methods: A cluster analysis of neurological symptoms was performed in a retrospective cohort of 56 CASPR2-Ab patients. In parallel, we studied immunological features and HLA.

Results: Cluster analysis distinguished those with predominant limbic symptoms (n=29/56) from those with peripheral nerve hyperexcitability (PNH; n=27/56). In the limbic-prominent group, limbic features were either isolated (LE/-; 18/56, 32.1%) or combined with extra-limbic symptoms (LE/+; 11/56, 19.6%). Those with PNH had either mild PNH isolated or co-occurring with limbic symptoms (PNH/-; 11/56, 19.6%); or severe PNH accompanied by extra-limbic involvement (PNH/+; 16/56, 28.6%), resembling historical MoS descriptions. LE/- and LE/+ patients shared immunological and genetic characteristics, justifying considering them as a single entity (LE). HLA-DRB1*11:01 was carried more frequently by LE (94.0%) compared to PNH/- (40.0%, p=0.048) and PNH/+ (0.0%, p=0.003) patients. CASPR2-Ab positivity in CSF was more frequent in LE (93.1%) than in PNH/- (57.1%, p=0.04) and PNH/+ (0.0%, $p=3.4x10^{-8}$) patients. CASPR2-Ab serum values were higher in LE (median 1:40960, range 1:10240-1:81920) than in PNH/- (1:160, 1:20-1:40960; p=0.002) and PNH/+ (1:3840, 1:40-1:20480; $p=1.5x10^{-5}$) patients. Only PNH/+ patients had malignant thymoma (87.5%, $p=4.1x10^{-10}$), serum LGI1-Ab (66.7%, $p=2x10^{-6}$), and myasthenia gravis (50%, $p=3.4x10^{-5}$).

Interpretation: Clinical, immunological, and genetic characteristics of CASPR2-Ab patients support the existence of 3 major disorders (LE, NMT, and MoS), suggesting distinct etiopathogeneses.

Introduction

Antibodies against contactin-associated protein-like 2 (CASPR2-Ab) are found in patients with limbic encephalitis (LE), acquired neuromyotonia (NMT), and Morvan syndrome (MoS) (Lancaster et al. 2011; Irani et al. 2010). While LE is a pure central nervous system (CNS) disorder characterized by temporal lobe seizures and anterograde amnesia (Malter et al. 2014; C. G. Bien et al. 2017; Joubert et al. 2016); MoS combines CNS symptoms such as confusion, hallucinations and insomnia, with severe dysautonomia and features of peripheral nerve hyperexcitability (PNH) (Irani et al. 2012). However, modern MoS descriptions are lacking and its definition remains vague (Lancaster et al. 2011). By contrast, NMT, or Isaacs' syndrome, is a pure PNH syndrome combining motor, autonomic and pain symptoms (A. Vincent et al. 2018). Interestingly, LE and NMT syndromes can be found associated in a minority of patients with CASPR2-Ab, and it is not clear whether these cases should be labeled as MoS (A. Vincent et al. 2018; Roger, Alliez, and Roger 1953; van Sonderen, Ariño, et al. 2016). In addition, other symptoms, such as ataxia, hyperkinetic movement disorders and weight loss, have been recently reported in CASPR2-Ab cohorts (van Sonderen, Ariño, et al. 2016; Becker et al. 2012; Balint et al. 2013; Joubert et al. 2016). Moreover, a recent case series suggested that instead of well-delineated syndromes, CASPR2-Ab associate with a set of core symptoms forming random combinations (van Sonderen, Ariño, et al. 2017). Interestingly, HLA (human leukocyte antigen) DRB1*11:01 has been linked to CASPR2-Ab disease, suggesting common immune mechanisms in all CASPR2-Ab patients (S. Binks et al. 2018). It, however, still remains unclear whether neurological symptoms combine randomly in CASPR2-Ab patients, or if specific neurological syndromes can be defined. Systematic studies of the distribution of neurological symptoms among CASPR2-Ab patients are therefore of major importance in order to establish a clear

classification of the diseases and provide better insight into their underlying mechanisms. Herein, we used cluster analysis to explore the distribution of symptoms in a cohort of CASPR2-Ab patients. In addition, we studied whether the immunological and genetic characteristics of the patients differed according to clinical presentations.

Methods

Subjects

All patients with CASPR2-Ab found in serum and/or cerebrospinal fluid (CSF) in our center from July 2010 to November 2018 were identified and the clinical and paraclinical features retrospectively collected from hospital charts (Table 1). Patients without enough data were excluded from the analysis. Written consent was obtained from all patients, and the study was approved by the Institutional Review Board of the Université Claude Bernard Lyon 1 and Hospices Civils de Lyon.

Table 1. List and definition of the clinical and paraclinical features.

Features	Basis of the scoring	Comments
Sex	Clinical descriptions	
Age*	Clinical descriptions	
Memory impairment	Clinical descriptions	Confirmed by cognitive tests
Dysexecutive syndrome	Clinical descriptions	Confirmed by cognitive tests
Temporal lobe epilepsy	Clinical descriptions	At least 2 clinical seizures with no lesional cause, with clinical and/or electrophysiological evidence suggesting a mesial temporal lobe origin
Behavioral disturbances	Clinical descriptions	Including confusional state
Cerebellar ataxia - permanent	Clinical descriptions	
Cerebellar ataxia - paroxysmal	Clinical descriptions	
Altered general state	Clinical descriptions	Asthenia and/or weight

		loss and/or anorexia
Weight loss	Clinical descriptions	At least 5 kg, or, if not quantified, qualified as "major" or "important"
Hyperkinetic movement disorders	Clinical descriptions	Myoclonus, dyskinesia, chorea-like movements
Insomnia - non characterized	Clinical descriptions	Patients reports difficulty to sleep with no further specifications
Major insomnia	Clinical descriptions	Patient reports complete or near-complete loss of sleep
Nocturnal hallucinations	Clinical descriptions	
*Agrypnia excitata**	Polysomnography	Diagnosis made by sleep expert
Sleep apnea*	Polysomnography	Diagnosis made by sleep expert
Peripheral nerve hyperexcitability	Clinical descriptions	fasciculations, myokimia, cramps with or without ENMG correlate
Functional impairment due to PNH motor symptoms	Clinical descriptions	Involuntary muscular contractions with moving of segments of limb impairing walk or basic activities
Generalized PNH motor symptoms	Clinical descriptions	4 limbs ± trunk
Severe signs of PNH on ENMG	ENMG study	Multiplets, and/or afterdischarges, and/or complex repetitive discharges, and/or myokimic discharges, and/or neuromyotonic discharges - Diagnosis made by expert electromyographists
Dysautonomia	Clinical descriptions	
Severe dysautonomia	Clinical descriptions	Profuse hypersudation (several changes per day), or orthostatism-independant blood pressure fluctuations, or postural syncopes, or intestinal pseudo-obstruction
Neuropathic pain	Patient's complaint	Type and topography of pain suggesting neuralgic pain
Malignant thymoma*	Histology	

Myasthenia gravis	ENMG	Diagnosis made by expert electromyographists
Dyspnea	Clinical descriptions	
Abnormal MRI*	MRI study	Medial temporal lobe hypersignal
Abnormal EEG*	EEG study	Abnormal rhythms, periodic patterns and epileptiform abnormalities
Inflammatory CSF*	CSF analysis	Defined as pleocytosis >5 cells and/or increased proteinorachia and/or presence of OCB

*Variables not included in the classification analysis.

Abbreviations: CSF, cerebrospinal fluid; EEG, electroencephalography; ENMG, electroneuromyography; MRI, magnetic resonance imaging; OCB, oligoclonal bands; PNH, peripheral nerve hyperexcitability.

Antibody detection

CASPR2-Ab and antibodies against leucine-rich glioma-inactivated 1 protein (LGI1-Ab) were detected as previously described by immunohistofluorescence on rat brain sections and a cell-based assay (CBA) (Joubert et al. 2016; Navarro et al. 2016). Titration of CASPR2-Ab in serum was done by end-point dilution using CBA on HEK293-cells transfected with CASPR2-coding plasmids; titers were determined as the lowest dilution with positive signal on transfected HEK293 cells.

HLA analysis

HLA genotyping was performed in peripheral blood using next generation sequencing (NGS) technology as previously described(Lange et al. 2014), using the MiSeq sequencer system (Illumina, San Diego, CA, US) in 30 CASPR2-Ab patients with available DNA. HLA genotyping was reported at a six-digit level whenever technically possible. Once the genotypes were obtained, the most likely haplotypes according to ethnical origin were selected based on the public database HaploStats of the National Marrow Donor Program

(NMDP). An ethnically matched sample of 300 healthy controls was provided by the HLA Laboratory from the French Blood Service (EFS Auvergne Rhone Alpes) with their genotypes at four-digit level for class I HLA (A, B, C), and DRB1 and DQB1 for class II HLA, also obtained by NGS technology (Omixon, Budapest, Hungary).

Statistical analysis

We selected 22 features of interest, including clinical features and signs of PNH on electroneuromyographic (ENMG) recordings that were categorized into binary variables (presence/absence) and summarized as numbers and percentages. Other 7 variables such as age, the presence of a malignant thymoma, polysomnographic recordings, brain magnetic resonance imaging (MRI), electroencephalogram (EEG), and CSF analysis, were excluded from classification analysis. First, non-supervised analyses were performed using hierarchical clustering with Jaccard distance, and correspondence factor analysis. Then, supervised analysis using the random forest method was performed in order to identify variables with the greatest importance for clustering. The features of interest were introduced in the random forest (ntree=1000, mtry=6). Thereafter, diagnostic test results (e.g. brain MRI, CSF analysis, CASPR2-Ab titers, HLA genotypes) were compared between the groups that had been identified through classification analysis. Categorical variables were first compared with the Fisher's exact test (two-tailed), and differences in CASPR2-Ab serum titers were evaluated with Mann-Whitney U test. Pairwise comparisons were then performed using the Holm procedure whenever statistically significant.(Holm 1979) Bonferroni´s method was used for correction for multiple allelic comparisons in HLA analysis, multiplying the p value by the number of alleles for each locus. Corrected $p<0.05$ were considered significant. Odds ratio (OR) with 95% confidence interval (CI) was used as a measure of the strength of associations.

All analyses were performed using the R software (R Core Team, 2014. R: A language

and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/) and SPSS software package version 25.0 (IBM Corp, Armonk, NY, US).

Results

Demographic features and ancillary tests

A total of 79 patients with CASPR2-Ab were diagnosed from July 2010 to November 2018 in the French Reference Center of Paraneoplastic Neurological Syndromes and Autoimmune Encephalitis. Twenty-three (29.0%) patients were excluded from analysis due to insufficient data (Fig 1). Among the 56 patients analyzed, 46 were male (82.1%), and median age was 61 years (range: 27-82). Polysomnographic studies were performed in 15/56 (26.8%) of the patients, demonstrating *agrypnia excitata* in 6/15 (40.0%) patients, and obstructive sleep apnea in 9/15 (60.0%) patients. ENMG was performed in 35/56 (62.5%) patients, including 28 with PNH symptoms (cramps, fasciculations, and/or myokimia). Needle ENMG examination found spontaneous motor unit activities (i.e. fasciculations potentials, multiplets, neuromyotonic discharges, myokimic discharges, or complex repetitive discharges) in 25/28 (89.3%) patients, including features considered as severe in 21/28 (75.0%). Brain MRI found mesial temporal lobes signal changes typical of LE in 18/44 (40.9%) patients, consistent in all cases with the presence of limbic symptoms. EEG was reported as abnormal in 22/44 (50.0%) patients, including abnormal focal or generalized slowness, inter-critical epileptiform activity, and electroclinical seizures. Inflammation of CSF was found in 26/47 (55.3%) patients.

Classification analysis

We used non-supervised classification analysis to categorize our 56 patients based on similarities of symptoms' combinations (Fig 1); those with limbic symptoms (i.e. anterograde amnesia and temporal lobe epilepsy) were distinguished from those with PNH symptoms, as 27/31 (87.1%) of the patients with temporal lobe epilepsy and/or memory impairment were clustered separately from the 27/28 (96.4%) of patients with PNH symptoms.

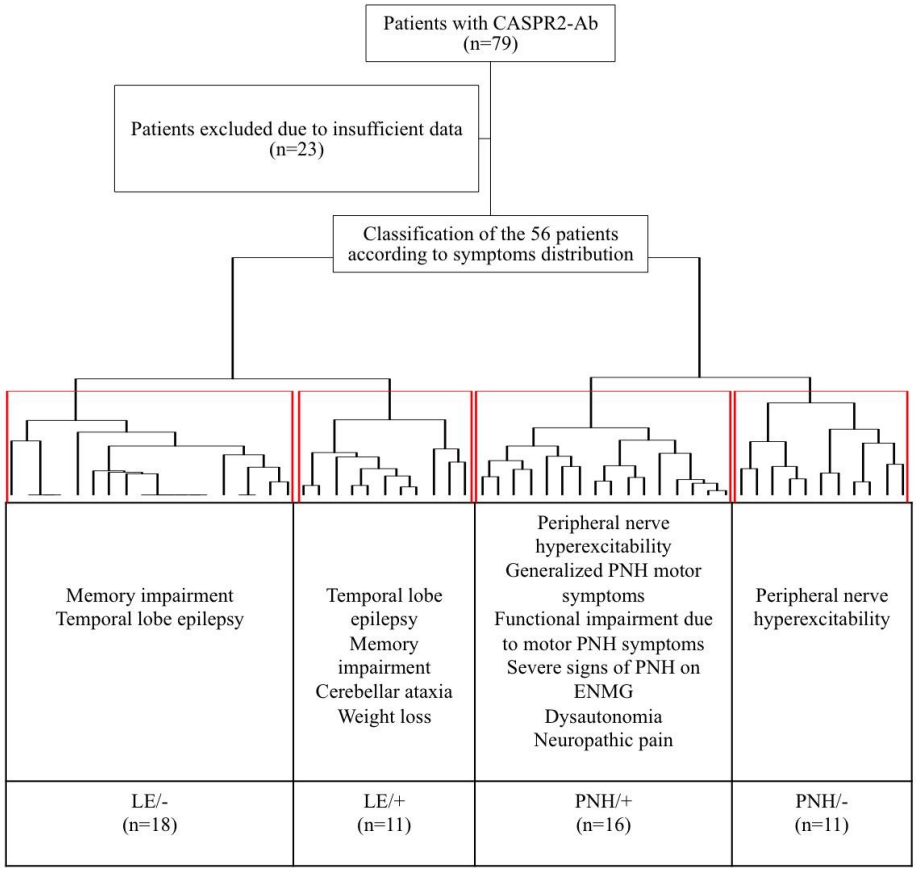

Figure 1. Flow chart presenting the study design and the classification analysis.
Classification analysis of the distributions of the symptoms among patients with anti-CASPR2-Ab determined 4 patient groups. The main symptoms in the cluster analysis for each group are indicated below. *Abbreviations: CASPR2-Ab, anti-CASPR2 antibodies; ENMG, electroneuromyography; LE, limbic encephalitis; PNH, peripheral nerve hyperexcitability.*

Moreover, within the limbic-predominant group of patients (29/56, 51.8%), classification analysis grouped together 11 patients (11/29, 37.9%; 11/56, 19.6% of the overall cohort) who had frequent additional non-limbic CNS symptoms, including cerebellar

ataxia (permanent in 8/11, 72.7%; and paroxysmal in 6/11, 54.5%) and hyperkinetic movement disorders (4/11, 36.4%), as well as dysautonomia (3/11, 27.3%) and weight loss (8/11, 72.7%). Extra-limbic symptoms were uncommon in the 18 patients from the rest of the limbic-predominant group (18/29, 62.1%; 18/56, 32.1% of the overall cohort), being present in only 5/18 (27.8%) patients (permanent ataxia, n=1; paroxysmal ataxia, n=2; hyperkinetic movement disorders, n=1; PNH symptoms, n=1). These groups were therefore labeled LE/- (isolated limbic encephalitis, n=18), reflecting the prominent isolated limbic involvement, and LE/+ (extensive limbic encephalitis, n=11), in order to emphasize the frequent association with extra-limbic features.

Among the 27 patients with predominant PNH features (27/56, 48.2%), classification analysis clustered 16/27 patients (59.3%; 16/56, 28.6% of the overall cohort) with severe PNH, as indicated by ENMG signs (15/15, 100% of those performed), functional impairment due to motor PNH signs (12/16, 75.0%), generalized PNH motor symptoms (13/16, 81.3%), neuropathic pain (12/16, 75.0%), and severe dysautonomia (11/16, 68.8%). All 16 patients had additional non-PNH symptoms, including non-limbic CNS involvement such as hyperkinetic movement disorders (8/16, 50.0%), permanent cerebellar ataxia (7/16, 43.8%), sleep disorders such as nocturnal hallucinations (7/16, 43.8%), any kind of insomnia (9/16, 56.2%) or proven *agrypnia excitata* (5/6, 83.3% from those with polysomnographic recordings), behavioral disorder (6/16, 37.5%), weight loss (9/16, 56.2%), and dyspnea (8/56, 50.0%); no patient had limbic symptoms. In the remaining 11/27 patients with predominant PNH (40.7%; 11/56, 19.6% of the overall cohort), PNH was less severe (severe PNH ENMG signs in only 6/11, 54.5%; functional impairment due to motor PNH signs in 1/11, 9.1%; generalized PNH signs in 4/11, 36.4%; severe dysautonomia in 1/11, 9.1%; neuropathic pain in 6/11, 54.5%), and limbic symptoms were seen in 4/11 patients (36.4%), reflecting the presence of patients with the association of limbic and PNH features. These groups were

therefore labeled, PNH/- in order to reflect mild and usually isolated PNH symptoms (n=11), and PNH/+ to reflect the association of severe PNH with extra-limbic CNS involvement (n=16).

There were 5 patients of the cohort with combined PNH and limbic symptoms; they were distributed between the LE/- (n=1) and PNH/- (n=4) groups. Malignant thymoma and myasthenia gravis were found only in the PNH/+ group, (14/16=87.5%, $p=4.1x10^{-10}$; and 8/16=50.0%, $p=3.4x10^{-5}$; respectively). No other type of tumor was observed.

Table 2 presents the distribution of all collected variables and Table 3 highlights the prominent clinical features in each group. Supervised random forest analysis identified PNH symptoms, severe PNH signs on ENMG, altered general state, and weight loss, as the most important variables for classifying the patients into the 4 diagnostic groups (Fig 2).

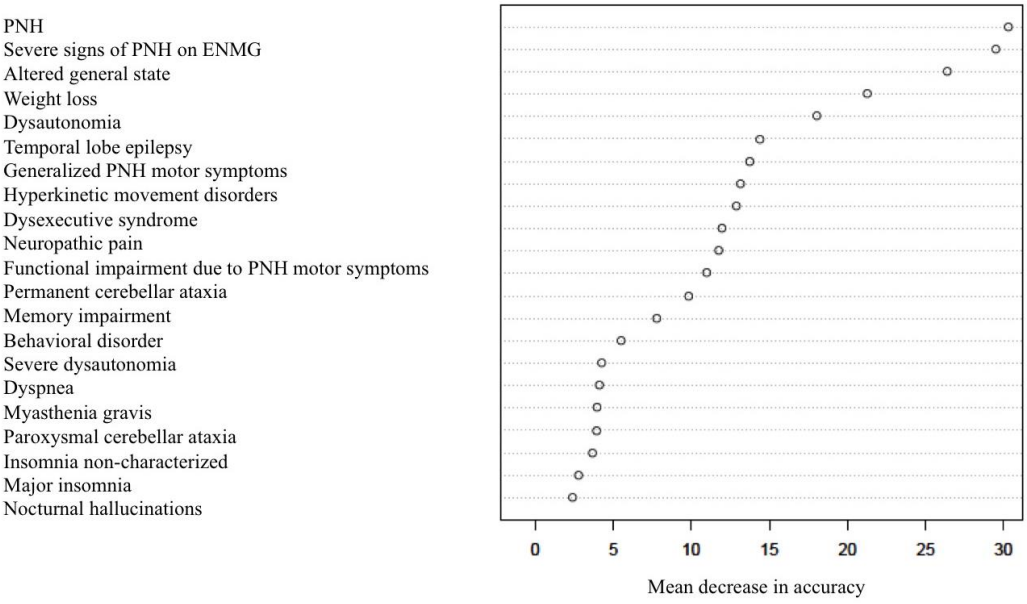

Figure 2. Random forest variable importance factor for mean accuracy.

Random forest analysis identified peripheral nerve hyperexcitability, altered general state, severe PNH signs on ENMG, and weight loss, as the most important features for classification. *Abbreviations: ENMG, electroneuromyography; PNH, peripheral nerve hyperexcitability.*

Table 2. Distribution of the clinical and paraclinical features in the overall cohort and the clinical groups defined in the cluster analysis.

	Total n=56 (%)	LE/- n=18 (32.1)	LE/+ n=11 (19.6)	PNH/+ n=16 (28.6)	PNH/- n=11 (19.6)
Male	46 (82.1)	17 (94.4)	11 (100)	12 (75)	6 (54.5)
Median age (range)	61 (27-82)	66 (53-78)	68 (57-82)	57.5 (29-78)	57 (27-71)
Memory impairment	31 (55.4)	16 (88.9)	11 (100)	0 (0.0)	4 (36.4)
Dysexecutive syndrome	22 (39.3)	14 (77.8)	7 (63.6%)	1 (6.2)	0 (0.0)
Temporal lobe epilepsy	31 (55.4)	17 (94.4)	10 (90.9)	0 (0.0)	4 (36.4)
Behavioral disorder	17 (30.4)	4 (22.2)	7 (63.6)	6 (37.5)	0 (0.0)
Permanent ataxia	17 (30.4)	1 (5.6)	8 (72.7)	7 (43.8)	1 (9.1)
Paroxysmal ataxia	10 (17.9)	2 (11.1)	6 (54.5)	0 (0.0)	2 (18.2)
Altered general state	21 (37.5)	0 (0.0)	8 (72.7)	12 (75.0)	1 (9.1)
Weight loss	18 (32.1)	0 (0.0)	8 (72.7)	9 (56.2)	1 (9.1)
Hyperkinetic movement disorders	14 (25.0)	1 (5.6)	4 (36.4)	8 (50.0)	1 (9.1)
Insomnia- non characterized	5 (8.9)	0 (0.0)	0 (0)	4 (25.0)	1 (9.1)
Major insomnia	6 (10.7)	0 (0.0)	1 (9.1)	5 (31.2)	0 (0.0)
Nocturnal hallucinations	7 (12.5)	0 (0.0)	0 (0.0)	7 (43.8)	0 (0.0)
*Agrypnia excitata**	6 (40.0)	0 (0.0)	1 (33.3)	5 (83.3)	0 (0.0)
Sleep apnea*	9 (60.0)	2 (100)	3 (100)	1 (16.7)	3 (75.0)
PNH	28 (50.0)	1 (5.6)	0 (0.0)	16 (100.0)	11 (100)
Functional impairment due to PNH motor symptoms	13 (23.2)	0 (0.0)	0 (0.0)	12 (75.0)	1 (9.1)
Generalized PNH motor symptoms	18 (32.1)	1 (5.6)	0 (0.0)	13 (81.3)	4 (36.4)
Severe signs of PNH on ENMG*	21 (60.0)	0 (0.0)	0 (0.0)	15 (100.0)	6 (54.5)
Dysautonomia	21 (37.5)	0 (0.0)	3 (27.3)	15 (93.8)	3 (27.3)
Severe dysautonomia	14 (25.0)	0 (0.0)	2 (18.2)	11 (68.8)	1 (9.1)
Neuropathic pain	21 (37.5)	2 (11.1)	1 (9.1)	12 (75.0)	6 (54.5)
Malignant thymoma	14 (25.0)	0 (0.0)	0 (0.0)	14 (87.5)	0 (0.0)
Myasthenia gravis	8 (14.3)	0 (0.0)	0 (0.0)	8 (50.0)	0 (0.0)
Dyspnea	8 (14.3)	0 (0.0)	0 (0.0)	8 (50.0)	0 (0.0)
Abnormal MRI*	18 (40.9)	12 (70.6)	3 (37.5)	0 (0.0)	3 (50.0)
Abnormal EEG*	22 (50.0)	11 (61.1)	5 (45.5)	3 (33.3)	3 (50.0)
Inflammatory CSF*	26 (55.3)	14 (82.4)	8 (72.7)	1 (7.7)	3 (50.0)

*Polysomnographic recordings N=15, ENMG N=35, MRI N=44, EEG N=44, and CSF N=47. Proportions (%) are expressed based on this number of patients instead of the total cohort.
Abbreviations: CSF, cerebrospinal fluid; EEG, electroencephalography; ENMG, electroneuromyography; LE, limbic encephalitis; MRI, magnetic resonance imaging; PNH, peripheral nerve hyperexcitability.

Table 3. Description of the prominent features (seen in at least 50% of the patients) included in the classification analysis that statistically define the 4 clinical groups.

Groups	LE/- n=18	LE/+ n=11	PNH/+ n=16	PNH/- n=11
Prominent features (%)				
Memory impairment	88.9	100		
Temporal lobe epilepsy	94.4	90.9		
Dysexecutive syndrome	77.8	63.6		
Behavioral disorders		63.6		
Permanent cerebellar ataxia		72.7		
Paroxysmal cerebellar ataxia		54.5		
Altered general state		72.7	75.0	
Weight loss		72.7	56.2	
PNH			100	100
Severe signs of PNH on ENMG			100	54.5
Generalized motor symptoms			81.3	
Functional impairment due to PNH motor symptoms			75.0	
Dysautonomia			93.8	
Severe dysautonomia			68.8	
Neuropathic pain			75.0	54.5
Hyperkinetic movement disorders			50.0	
Dyspnea			50.0	

Abbreviations: ENMG, electroneuromyography; LE, limbic encephalitis; PNH, peripheral nerve hyperexcitability.

Regarding the ancillary tests not included in the cluster analysis, the presence of mesial temporal signal changes in brain MRI was significantly more frequent in LE/- patients compared to PNH/+ patients (12/17, 70.6%; vs. 0/13, 0.0%; $p=5x10^{-4}$); there was no significant difference between the other groups (12/17, 70.6% LE/-, vs. 3/8, 37.5% LE/+, p=0.5; 12/17, 70.6% LE/- vs. 3/6, 50.0% PNH/-, p=1; 3/8, 37.5% LE/+, vs. 0/13, 0.0%

PNH/+, p=0.1; 3/8, 37.5% LE/+, vs. 3/6, 50.0% PNH/- p=1; 0/13, 0.0% PNH/+, vs. 3/6, 50.0% PNH/-, p=0.1). CSF inflammation was significantly more frequent in LE/- and LE/+ patients (14/17, 82.4%; and 8/11, 72.7%; respectively) compared to PNH/+ patients (1/13, 7.1%; $p=6x10^{-4}$ and p=0.01, respectively) but not to PNH/- patients (3/6, 50.0%; p=0.8 and p=1, respectively). Brain MRI abnormalities (3/6, 50.0%) and CSF inflammation (3/6, 50.0%) in the PNH/- group were attributable to the 4 patients of this group who had overlapping limbic and PNH features.

Immunological features

All but 1 of the sera tested for CASPR2-Ab (n=52: 16 LE/-, 9 LE/+, 16 PNH/+, 11 PNH/-) were found positive; there was no significant difference in the detection of serum CASPR2-Ab between groups (p=0.5). Among all tested CSF, 31/48 (64.6%) samples were positive for CASPR2-Ab. CSF detection rates of CASPR2-Ab did not differ between LE/- and LE/+ groups (16/18 vs. 11/11, p=0.5); when comparing all LE patients (27/29, 93.1%) to PNH/- and PNH/+ patients, detection of CASPR2-Ab in CSF was significantly more frequent in LE patients than in those from both the PNH/- (27/29 vs. 4/7, p=0.04) and PNH/+ (27/29 vs. 0/12, $p=3.4x10^{-8}$) groups. In addition, CASPR2-Ab were more commonly found in the CSF of PNH/- than of PNH/+ patients (4/7 vs. 0/12, p=0.018); these were the 4 CSF-positive PNH/- patients who had overlapping PNH and limbic features. Serum LGI1-Ab were only detected in PNH/+ patients (10/15, 66.7%, $p=2x10^{-6}$) and only in those with malignant thymoma (10/13 vs. 0/37, $p=2.7x10^{-8}$). Among the 46 CSF also tested for LGI1, only 1 (0.02%) was positive at very low titers (end-point dilution, 1:20), belonging to a patient of the PNH/+ group.

Serum was still available for the establishment of CASPR2-Ab end-point dilution for 43 patients (13 LE/-, 9 LE/+, 12 PNH/+, 9 PNH/-). The median end-point dilution was

1:40960 (range: 1:10240-1:81920) for LE/-, 1:40960 (range: 1:10240-1:81920) for LE/+, 1:3840 (range: 1:40-1:20480) for PNH/+, and 1:160 (range: 1:20-1:40960) for PNH/-. Serum CASPR2-Ab titers were not significantly different between LE/- and LE/+ patients (p=0.7); they were significantly higher in all LE patients combined compared to PNH/+ ($p=1.5x10^{-5}$) and PNH/- groups (p=0.002); there was no significant difference between PNH/+ and PNH/- groups (p=0.7; Table 4).

Table 4. Immunological characteristics according to clinical phenotypes.

	LE/- and LE/+ (n=29)	PNH/+ (n=16)	PNH/- (n=11)	p value*
CASPR2-Ab serum; positivity/tested (%)	24/25 (96.0%)	16/16 (100%)	11/11 (100%)	p=0.5
CASPR2-Ab serum titers; median (range)	1:40960 (1:10240-1:81920)	1:3840 (1:40-1:20480)	1:160 (1:20-1:40960)	LE vs. PNH/+, $p=1.5x10^{-5}$ LE vs. PNH/-, p=0.002 PNH/+ vs. PNH/-, p=0.7
CASPR2-Ab CSF; positivity/tested (%)	27/29 (93.1%)	0/12 (0%)	4/7 (57.1%)	LE vs. PNH/+, $p=3.4x10^{-8}$ LE vs. PNH/-, p=0.04 PNH/+ vs. PNH/-, p=0.018
LGI1-Ab serum; positivity/tested (%)	0/25 (0.0%)	10/15 (66.7%)	0/10 (0.0%)	$p=2x10^{-6}$

*Statistical analysis: Fisher´s exact test for categorical variables and Mann-Whitney U test for CASPR2-Ab serum titers.

Abbreviations: CASPR2-Ab, anti-CASPR2 antibodies; CSF, cerebrospinal fluid; LE, limbic encephalitis; LGI1-Ab, anti-LGI1 antibodies; PNH, peripheral nerve hyperexcitability.

HLA genotyping

DNA was available for HLA genotyping for 30 patients, all were of Caucasian-origin, there were 28 males (93.3%), and the median age was 61.5 years (range: 28-73). The patients belonged respectively to groups LE/- (10/30, 33.3%), LE/+ (7/30, 23.3%), PNH/+ (8/30, 26.7%) and PNH/- (5/30, 16.7%). Of note, 7/8 (87.5%) of the PNH/+ patients genotyped had

a malignant thymoma, and 5/8 (62.5%) had serum LGI1-Ab.

HLA allele DRB1*11:01 was present in 20/30 patients (66.7%) vs. 55/300 (18.3%) in the healthy control group ($p=1x10^{-6}$, OR=8.909, 95% CI 3.95-20.097). DQB1*03:01:01 was found at a similar frequency due to linkage disequilibrium with DRB1*11:01 (22/30 vs. 100/300 in controls, $p=3x10^{-4}$, OR=5.5, 95% CI 2.365-12.792).

Among the DRB1*11:01 carriers, 19/20 (95.0%) carried DQB1*03:01 along with DQA1*05 when DQA1 was successfully genotyped (17/19); forming the extended haplotype DRB1*11:01-DQB1*03:01-DQA1*05. The remaining DRB1*11:01 patient (group LE/+) had the DRB1*11:01:02-DQB1*05:02:01-DQA1*01 haplotype. Individual HLA genotyping results are detailed in Table 5.

Table 5. HLA analysis and clinical classification in 30 patients with CASPR2-Ab.

Sex, age (years)	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1	HLA-DQA1	Group
M, 62	01:01:01	49:01:01	07:01:01	**11:01:01**	03:01:01	05:XX	LE/-
	03:02:01	51:01:01	15:02:01	**11:01:01**	03:01:01	05:XX	
M, 66	24:02:01	57:01:01	06:02:01	**11:01:01**	03:01:01	05:XX	LE/-
	02:01:01	18:01:01	05:01:01	**11:01:01**	03:01:01	05:XX	
M, 69	02:01:01	35:08:01	12:03:01	**11:01:01**	03:01:01	05:XX	LE/-
	24:02:01	38:01:01	12:03:01	11:04:01	03:01:01	05:XX	
M, 53	24:02:01	39:06:02	07:02:01	**11:01:02**	03:01:01	XX	LE/-
	01:01:01	57:01:01	06:02:01	03:01:01	02:01:01	XX	
M, 66	02:01:01	49:01:01	07:01:01	**11:01:01**	03:01:01	05:XX	LE/-
	23:01:01	49:01:01	07:01:01	**11:01:01**	03:01:01	05:XX	
M, 61	02:01:01	39:01:01	05:01:01	**11:01:01**	03:01:01	05:05:01	LE/-
	02:01:01	44:02:01	01:02:01	13:01:01	06:03:01	01:XX	
M, 71	02:01:01	15:01:01	03:03:01	**11:01:01**	03:01:01	05:XX	LE/-
	26:01:01	45:01:01	06:02:01	**11:01:01**	03:01:01	05:XX	
M, 72	03:01:01	07:02:01	07:02:01	15:01:01	06:02:01	01:02:01	LE/-
	24:02:01	52:01:01	12:02:02	04:01:01	03:02:01	03:01:01	
M, 66	68:01:02	44:02:01	07:04:01	**11:01:01**	03:01:01	05:XX	LE/-
	03:01:01	07:02:01	07:02:01	13:01:01	06:03:01	01:03:01	
M, 61	02:01:01	18:01:01	07:04:01	**11:01:01**	03:01:01	05:XX	LE/-
	23:01:01	49:01:01	07:01:01	04:05:01	03:02:01	03:XX	

M, 71	02:01:01	51:01:01	15:02:01	**11:01:01**	03:01:01	05:XX	LE/+
	24:02:01	35:01:01	04:01:01	08:02:01	04:02:01	04:01:01	
M, 60	23:01:01	49:01:01	07:01:01	**11:01:01**	03:01:01	05:XX	LE/+
	32:01:01	44:02:01	05:01:01	13:01:01	06:03:01	01:03:01	
M, 72	01:01:01	XX	06:02:01	**11:01:01**	03:01:01	XX	LE/+
	02:01:01	XX	16:01:01	11:02:01	03:19:01	XX	
M, 69	29:02:01	07:05:01	15:05:01	**11:01:02**	05:02:01	01:02:01	LE/+
	32:01:01	27:05:02	02:02:02	04:05:01	02:XX	03:XX	
M, 68	01:01:01	07:01:01	08:02:01	**11:01:01**	03:01:01	05:XX	LE/+
	01:01:01	08:01:01	14:01:01	07:01:01	02:XX	02:01:01	
M, 62	24:02:01	40:02:01	02:02:02	**11:01:01**	03:01:01	05:XX	LE/+
	02:01:01	44:03:01	04:01:01	07:01:01	02:XX	02:01:01	
M, 57	24:02:01	56:01:01	01:02:01	**11:01:01**	03:01:01	05:XX	LE/+
	29:02:01	40:02:01	02:02:02	**11:01:01**	03:01:01	05:XX	
F, 58	02:01:01	44.04:01	02:02:02	16:01:01	05:02:01	01:02	PNH/+
	23:01:01	08:01:01	07:01:01	03:01:01	02:01:01	05:01:01	
M, 48	02:01:01	44:02:01	05:01:01	04:01:01	03:01:01	03:XX	PNH/+
	24:02:01	50:01:01	06:02:01	04:04:01	03:02:01	03:XX	
M, 60	02:01:01	58:01:01	07:01:01	**11:01:01**	03:01:01	05:XX	PNH/+
	24:02:01	18:01:01	12:03:01	11:04:01	03:01:01	05:XX	
M, 65	02:01:01	27:05:02	01:02:01	01:03:01	03:01:01	05:XX	PNH/+
	30:01:01	13:02:01	06:02:01	04:08:01	03:01:01	03:01:01	
M, 41	02:01:01	39:01:01	12:03:01	16:01:01	05:02:01	01:02	PNH/+
	24:02:01	49:01:01	07:01:01	11:04:01	03:01:01	05:XX	
M, 43	29:02:01	07:02:01	07:02:01	15:01:01	06:02:01	01:02:01	PNH/+
	32:01:01	08:01:01	07:01:01	03:01:01	02:01:01	05:01:01	
M, 73	02:01:01	15:01:01	03:03:01	11:01:01	03:01:01	05:XX	PNH/+
	29:02:01	44:03:01	16:01:01	07:01:01	02:XX	02:01:01	
M, 29	02:01:01	35:01:01	04:01:01	03:01:01	02:XX	05:01:01	PNH/+
	29:02:01	44:03:01	16:01:01	07:01:01	02:01:01	02:01:01	
F, 37	01:01:01	49:01:01	07:01:01	04:05:01	03:02:01	03:XX	PNH/-
	11:01:01	55:01:01	03:03:01	13:01:01	06:03:01	01:03:01	
M, 28	11:01:01	44:02:01	05:01:01	04:01:01	03:02:01	03:XX	PNH/-
	03:01:01	56:01:01	07:02:01	01:01:01	05:01:01	01:01:01	
M, 39	24:02:01	49:01:01	07:01:01	**11:01:01**	03:01:01	05:XX	PNH/-
	29:02:01	44:03:01	16:01:01	07:01:01	02:XX	02:01:01	
M, 58	02:01:01	44:05:01	02:02:02	01:01:01	05:01:01	01:01:01	*PNH/-
	24:02:01	15:01:01	03:03:01	01:03:01	05:01:01	01:01:01	
M, 68	11:01:01	18:01:01	07:01:01	**11:01:01**	03:01:01	05:XX	*PNH/-
	29:02:01	44:03:01	16:01:01	07:01:01	02:XX	02:01:01	

For a given patient, each haplotype is shown in a separate row. Alleles are reported at six-digit level, unless it was not technically possible (XX). DRB1*11:01 is highlighted in bold. *****PNH/- indicates patients presenting also with limbic features.

Abbreviations: F, female; HLA, human leukocyte antigen; LE, limbic encephalitis; M, male; PNH, peripheral nerve hyperexcitability.

Notably, most DRB1*11:01 carriers (19/20, 95.0%) did not have a tumor, while a malignant thymoma was found in 6/10 (60.0%) patients who did not carry this allele (p=0.002, OR=0.035, 95% CI 0.003-0.0378) demonstrating prominent association with non-paraneoplastic CASPR2-Ab disease. In terms of clinical presentations, DRB1*11:01 was carried by most LE/+ and LE/- patients (16/17=94.1%) compared to only 25.0% (2/8) of PNH/+ patients (p=0.003, OR=3.765, 95% CI 1.127-12.576), and 40.0% of PNH/- patients (2/5, p=0.048, OR=2.353, 95% CI 0.799-6.929). The frequency of DRB1*11:01 among PNH/+ (25.0%) and PNH/- (40.0%) patients was not significantly different to that found in the healthy control group (18.3%; p=0.6 and p=0.2, respectively), but significantly higher in LE patients compared to the healthy control group (94.1% vs. 18.3%; $p=1.3x10^{-10}$). None of the LGI1-Ab-positive patients carried the DRB1*07:01 allele.

Discussion

CASPR2-Ab are classically associated with acquired NMT, MoS and LE; but recent studies suggested that their clinical spectrum is in fact more extensive and borders among them may be blurred (Irani et al. 2010; van Sonderen, Ariño, et al. 2016). The present study found that symptoms of CASPR2-Ab patients do not associate randomly; but they form preferential combinations instead. In particular, limbic and PNH symptoms tend to cluster separately and their overlap represents only a minority of the patients (Fig 3). On the one hand, limbic symptoms can either be the only CNS features in the patients (LE/- group), or associate with non-limbic symptoms such as weight loss, cerebellar ataxia, or hyperkinetic movement disorders (LE/+ group), thereby confirming that the phenotype of CASPR2-Ab LE extends beyond limbic symptoms. On the other hand, a group of patients who had mild PNH

symptoms (PNH/- group) corresponded to patients with NMT, which can appear isolated or combined with limbic symptoms in a minority of patients. In addition, we identified a group of patients with PNH features that were considered to be severe (motor functional impairment, generalized distribution, pain, and severe autonomic disturbances) and with frequent non-limbic CNS symptoms including sleep disturbances, severe dysautonomia, and weight loss (PNH/+). Interestingly, this combination of symptoms is reminiscent of the historical descriptions of MoS,(Roger, Alliez, and Roger 1953) and this clinical presentation was strongly associated with an underlying malignant thymoma.

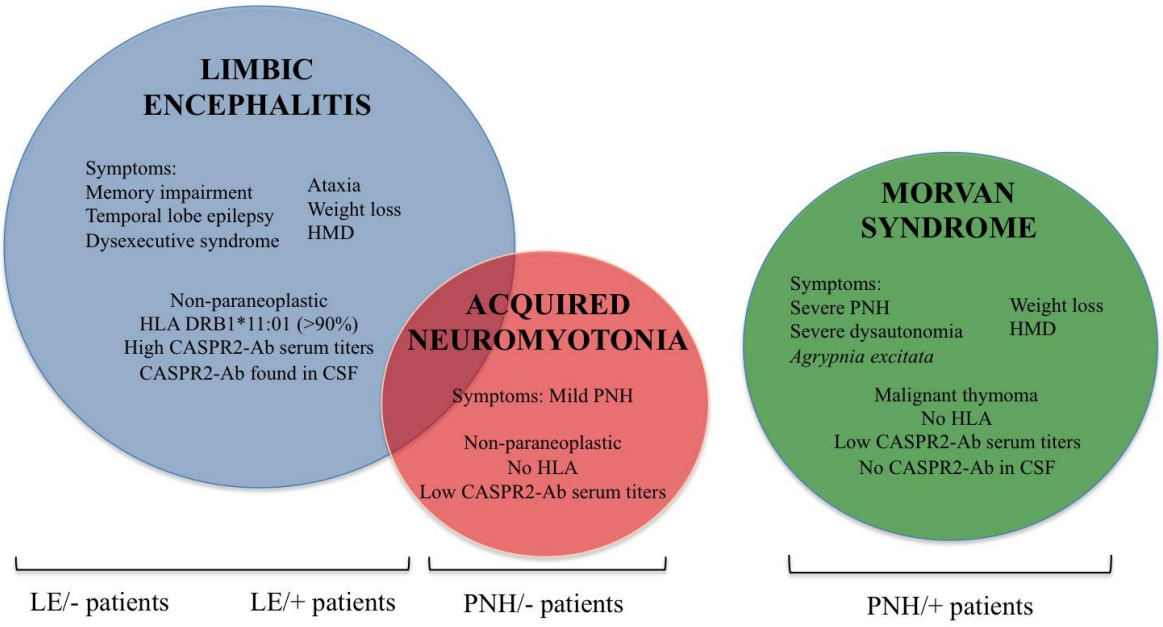

Figure 3. Clinic and immunogenetic features define three major CASPR2-Ab diseases with different pathogenesis.

Schematic features and distribution within the present study population of the three main CASPR2-Ab syndromes (limbic encephalitis, acquired neuromyotonia, Morvan syndrome), as suggest the results of the cluster analysis; a minority of patients had an association of NMT and LE features.

Abbreviations: HLA, human leukocyte antigen; HMD, hyperkinetic movement disorders; PNH, peripheral nerve hyperexcitability, CASPR2-Ab, anti-CASPR2 antibodies; CSF, cerebrospinal fluid.

The patients of the PNH/+ group were distinctive also regarding their genetic and immunological features. Although we confirm the strong association of anti-CASPR2 immunity with the HLA DRB1*11:01 allele in the LE/- and LE/+ groups, this association was absent in the PNH/+ group, suggesting this clinical presentation results from a different etiopathogeny. However, the number of patients studied for HLA was relatively small and we cannot exclude bias from a sampling effect. Further differentiating this group, PNH/+ patients had significantly lower serum titers of CASPR2-Ab compared to LE patients, in addition they did not present CASPR2-Ab in CSF, and commonly had myasthenia gravis and serum LGI1-Ab.

A sleep recording demonstrating *agrypnia excitata* is usually considered as the cornerstone of the diagnosis of MoS,(Liguori 2001; Lanuzza et al. 2012) but due to the retrospective collection of the data, systematic polysomnographic recordings were lacking to study this aspect herein. It is however remarkable that sleep disturbances were very common in PNH/+ patients in the present study and over 80% with polysomnographic recording had a classical pattern of *agrypnia excitata* including features such as reduction of spindles, reduction of total sleep time, and periods of type 1 non-REM sleep alternating with REM sleep.(Lugaresi, Provini, and Cortelli 2011) Notably, polysomnographic recording of 1 LE/+ patient complaining of major insomnia also displayed an *agrypnia* pattern. *Agrypnia excitata*, a rare sleep disorder observed also ~~i~~n fatal familial insomnia and delirium tremens, is thought to result from thalamo-limbic dysfunction (Lugaresi, Provini, and Cortelli 2011). Of note, obstructive sleep apnea syndrome was also frequently observed in patients outside of the PNH/+ group. This finding could be fortuitous and be due to the overrepresentation of middle-aged males, although it may also result from autonomic dysregulation.(Lombardi, Pengo, and Parati 2019) More systematic recordings will be necessary to fully apprehend the nature of the sleep disorders in the various CASPR2-Ab syndromes.

Although the PNH/+ patients have highly distinctive features reminiscent of MoS, such as particularly severe PNH symptoms, severe dysautonomia, *agrypnia*, weight loss, and association with malignant thymoma; we found that patients from the LE/+ group can have features resembling those of PNH/+ patients (i.e. weight loss, dysautonomia, and hyperkinetic movement disorders). Therefore, dysautonomia, weight loss, and central hyperexcitability might represent a phenotype partly shared between LE and MoS. Nevertheless, LE/+ patients had no PNH symptoms, and usually mild dysautonomia. In addition, LE/+ patients had immunological and genetic characteristics similar to LE/- instead of PNH/+ patients, suggesting LE/- and LE/+ patients share a common pathogenesis and should be considered as a single entity. We also confirm the previous findings that LE is associated with increased serum titers and increased CSF detection of CASPR2-Ab compared to NMT (PNH/-) and MoS (PNH/+) patients (Joubert et al. 2016; C. G. Bien et al. 2017). Interestingly, high CASPR2-Ab serum titers and CASPR2-Ab in CSF in PNH/-patients were only present in those with overlapping LE. Since the epitopes targeted by CASPR2-Ab are similar among patients, phenotype determination could depend in part on the site of CASPR2-Ab synthesis (Joubert et al. 2016).

Importantly, the differences in the immunological and genetic characteristics in the PNH/+ group compared to the LE and PNH/- patients are likely related to the increased frequency of malignant thymoma in PNH/+ patients. Although confirmation in larger cohorts is needed, patients with malignant thymoma herein did not carry more frequently than healthy population the HLA DRB1*11:01 allele linked to anti-CASPR2 immunity. Interestingly, an *in silico* study has shown that DQB1*03:01-DQA1*05:01 (which are in linkage disequilibrium with DRB1*11:01) heterodimer may be involved in CASPR2-derived peptides presentation (S. Binks et al. 2018). Therefore, two independent immunological pathways may be involved in CASPR2-Ab patients, depending on the presence or not of a malignant thymoma. In the

non-paraneoplastic cases, altered peptide presentation to CD4 T-cells mediated by class II HLA may allow evasion of thymic negative selection and/or peripheral self-tolerance mechanisms. Conversely, in patients with malignant thymoma breaking of tolerance is likely to result from abnormal T-cell maturation within the tumor microenvironment, implying a wider and less specific immune response. This is consistent with the frequent presence of myasthenia gravis and LGI1-Ab in patients with malignant thymoma, who, in addition, remarkably lacked the DRB1*07:01 allele recently linked to anti-LGI1 encephalitis (Kim et al. 2017; van Sonderen, Roelen, et al. 2017). Of note, anti-netrin-1 receptor autoantibodies also have been described mainly in paraneoplastic CASPR2-Ab disease, illustrating the wide spectrum of the immune response in thymoma-associated CASPR2-Ab patients.(Torres-Vega et al. 2017)

In conclusion, the present study shows how symptoms do not combine randomly in CASPR2-Ab patients, but confirms the existence of 3 different clinical phenotypes with only a minor overlap between LE and NMT, and MoS as the most distinctive group. Genetic (HLA DRB1*11:01 uniquely associated with LE), co-morbidities (mainly malignant thymoma in MoS), and immunological features support the classification into 3 major CASPR2-Ab diseases, and suggest distinct pathogenic mechanisms. Future studies are needed to clarify how immunological and neurobiological mechanisms determine clinical phenotypes in CASPR2-Ab patients.

Commentary.

In this study, we aimed at determining if the symptoms in CASPR2-Abs patients form preferential combinations instead of being random, as Van Sonderen and colleagues proposed (van Sonderen, Ariño, et al. 2016; 2017). To do so, we used the French cohort of CASPR2-Abs patients (with a now substantially increased headcount of 56 patients), and performed statistical cluster analysis of the neurological symptoms. In addition, we studied how immunological, HLA allele, and oncological association correlate with clinical phenotypes. The retrospective design of the study made that clinical data could not be exhaustively collected for every patient, with the risk of being unable to detect symptoms important for the classification (e.g. a specific sleep disorder, since not all patients with insomnia had a polysomnographic recording), and limited the amount of samples available for genetic or antibody testing. In spite of this, we observed that the patients' symptoms do not combine randomly, but instead form different clinical patterns, allowing to classify the patients into 3 groups: the patients with limbic encephalitis, the patients with neuromyotonia, and a group of patients with a mix of peripheral and central nervous system symptoms that we hypothesize corresponds to the Morvan syndrome patients. Similarly to the articles from Roger et al. and Irani et al., the prominent clinical features of the latter group were severe peripheral nerve hyperexcitability, severe dysautonomia, absence of limbic symptoms, sleep disorders corresponding to agrypnia excitata in sleep recordings, weight loss, and hyperkinetic movement disorders, with frequent association with thymoma. Thus, statistical cluster analysis of the symptoms' distribution makes a clear distinction between patients with limbic symptoms on the one hand, and patients with a clinical presentation compatible with Morvan syndrome on the other hand. In addition to emphasizing the existence of a distinctive group of patients corresponding to Morvan syndrome, our data improves the clinical presentation of CASPR2 autoimmune encephalitis. As others and we had reported, we confirm its extensive

clinical spectrum, some patients having, in addition to limbic symptoms, a variety of non-limbic signs, including ataxia, dysautonomia, and weight loss (Joubert et al. 2016; van Sonderen, Ariño, et al. 2017). In addition, our study emphasize the frequency of hyperkinetic movement disorders, which were under-recognized in previous studies (C. G. Bien et al. 2017; van Sonderen, Ariño, et al. 2016). Interestingly, the association of limbic symptoms and neuromyotonia was found in less than 10% of the patients. Furthermore, as Bien and colleagues and we had previously observed, serum CASPR2-abs levels were higher in limbic encephalitis patients compared to Morvan and neuromyotonia patients, and limbic encephalitis was associated with CASPR2-Abs CSF positivity, while in most neuromyotonia and Morvan patients CSF testing for CASPR2-Ab was negative (Joubert et al. 2016; C. G. Bien et al. 2017). These findings suggest that there are correlates between the characteristics of the immune activation and the neurological phenotype, which are not fully understood yet. In addition, we observed that most of the patients with an underlying malignant thymoma (all of them with Morvan syndrome) lacked the CASPR2-Ab-associated HLA DRB1*1101 allele, found in 94% of the non-paraneoplastic, limbic encephalitis patients. This may suggest different immunopathological pathways in thymoma patients compared to patients without a thymoma, although this has to be studied in larger cohorts. Overall, our findings support the existence of three distinct phenotypes among CASPR2-Abs patients, which are likely underlined by different pathophysiological processes.

PERSPECTIVES

CLINICAL SIGNIFICANCE

In the present PhD project, we address the issue of the clinical spectrum of CASPR2-Abs, with the aim of improving the characterization and classification of CASPR2-Abs patients. First of all, we observed that CASPR2-Abs are found in a specific population of patients - predominantly males in their sixth to eight decade - with a restricted number of signs and symptoms, confirming that CASPR2-Abs are biomarkers specifically associated with at least one specific autoimmune neurological syndrome. Moreover, our data demonstrate that the symptoms of the patients combine in a non-random fashion, supporting the existence of three distinct clinical patterns corresponding respectively to autoimmune limbic encephalitis, Morvan syndrome, and acquired neuromyotonia. Our data suggest that co-occurrence of two of these syndromes is possible (e.g. neuromyotonia and limbic encephalitis) even though it concerns only a minority of cases. In addition, our findings outline the main clinical features of each of these syndromes (Table II). Notably, we provide a detailed description of the clinical presentation of autoimmune limbic encephalitis in CASPR2-Abs patients. In accordance with findings from other groups, we observe that the clinical presentation of CASPR2 encephalitis is far more extensive that suggested by the initial reports of CASPR2-Ab patients and that one-third of the patients have non-limbic symptoms including ataxia, mild dysautonomia, weight loss, and movement disorders (Lancaster et al. 2011; Irani et al. 2010; van Sonderen, Ariño, et al. 2016; C. G. Bien et al. 2017). In addition, we report the first cases of autoimmune episodic ataxia, a novel symptom that affects exclusively CASPR2-Abs patients with autoimmune limbic encephalitis. Besides, we also study the outcomes of patients with CASPR2-Abs and autoimmune limbic encephalitis, demonstrating that they usually respond well to immunotherapy, although some patients are left with cognitive symptoms, ataxia, or epilepsy.

	Autoimmune Limbic Encephalitis	**Acquired Neuromyotonia**	**Morvan Syndrome**
Symptoms	**Temporal lobe epilepsy** - *93%* **Anterograde amnesia** - *93%* **Pure limbic syndrome** - *45%* **Limbic and extra -limbic** - *55%* Cerebellar ataxia - *31%* Weight loss - *28%* Movement disorders - *17%* Dysautonomia - *10%*	**Mild to severe signs of PNH** Motor symptoms - *100%* Neuralgic pain - *55%* Dysautonomia - *27%*	**Severe signs of PNH** Motor symptoms - *100%* Dysautonomia - *94%* Neuralgic pain - *75%* **Severe insomnia**- *70%* **Nocturnal hallucinations** - *50%* **Weight loss** - *56%* **Movement disorders** - *50%* **No temporal lobe epilepsy** **No anterograde amnesia**
Male predominance	97%	55%	75%
Oncological association	None	None	Malignant thymoma
Genetic Background	HLA DRB1*1101	Unclear	None
Immunological features	High CASPR2-Abs serum titers CASPR2-Abs in CSF	Low CASPR2-Abs serum titers	Low CASPR2-Abs serum titers Multiple autoantibodies: CASPR2, LGI1, AchR, Netrin-1R

Table II. French cohort of CASPR2-Abs patients: main clinical, oncological, and immunological features according to syndromes

Apart from autoimmune limbic encephalitis, we also provide the clinical presentation of our Morvan syndrome patients. We confirm that these patients have severe peripheral nerve hyperexcitability, severe autonomic symptoms, a severe sleep disorder including agrypnia excitata features, and no limbic symptoms such as amnesia or temporal lobe epilepsy. We also emphasize in Morvan syndrome patients the presence of hyperkinetic movement disorders and the association with malignant thymoma. Finally, we show correlations between the clinical phenotype and antibody production specificities, HLA variants, and oncological associations. Such correlations between clinical phenotypes and immunological and genetic characteristics suggest that depending on the patient, different pathophysiological mechanisms may be involved in anti-CASPR2 autoimmunity, explaining the clinical variability (Figure V). However, given the relatively small numbers of patients, in particular

of HLA-tested patients, these immunological-clinical correlations will need to be confirmed in other cohorts.

IMPLICATIONS FOR CLINICAL RESEARCH

Importantly, our results, by refining the clinical spectrum of CASPR2-Abs, pave the way for future clinical and basic research studies. From a clinical perspective, it will be important to describe in more details some aspects of the Morvan syndrome. For instance, although case reports, and our own data, suggest that agrypnia excitata is a cornerstone of Morvan syndrome, more than 60% of our Morvan syndrome patients, while complaining of insomnia, did not have their sleep recorded. Besides, we observed that sleep apnea syndrome was frequent in our cohort of CASPR2-Abs patients, but we were unable to determine if it was a chance association or reflected a specific aspect of the disease. Therefore, systematic studies focusing on the sleep disorders in CASPR2-Abs patients are needed. Another point is the issue of the hyperkinetic movement disorders, which we found in both autoimmune limbic encephalitis and Morvan syndrome patients. The exact nature, anatomical origin, and response to symptomatic treatments, of the movement disorders in CASPR2-Ab patients need to be studied. In addition, the outcomes of Morvan syndrome patients still remain to be assessed. Poor outcomes have been reported in the article by Irani and colleagues, contrasting with small cases series and case reports that found dramatic improvements after treatment with rituximab (Sadnicka et al. 2011; Irani et al. 2012; Weiss 2012; Ong et al. 2013).

IMPLICATIONS FOR BASIC RESEARCH

That CASPR2-Abs associate with three distinct syndromes raises the question of the factors that, besides the autoantibodies, determine phenotypes. So far, the pathogenic effects of CASPR2-Abs have been demonstrated mostly in *in vitro* models, with often discrepant results

(Saint-Martin et al. 2019; Patterson, Dalmau, and Lancaster 2018; Fernandes et al. 2019). Although it has been clearly demonstrated that CASPR2-Abs prevent the binding of TAG-1 to CASPR2, the downstream effects, in particular on glutamatergic receptors and potassium channels, are unclear (Saint-Martin et al. 2019; Patterson, Dalmau, and Lancaster 2018; Fernandes et al. 2019). Improving our understanding of the molecular disruptions caused by the binding of CASPR2-Abs onto CASPR2, at both the central and peripheral nervous systems levels, are essential. However, speculations about the effects of CASPR2-Abs are complicated by the fact that the roles of CASPR2 in the nervous system are still incompletely understood, especially at synapses.

Importantly, it is clear that the clinical variability is not due to differences in CASPR2-Abs epitope specificities, since CASPR2-Abs recognize the same epitopes in all patients regardless of the clinical presentation (Joubert et al. 2016). Our data show that serum titers, and CSF detection, of CASPR2-Abs, correlate with clinical presentation, suggesting clinical variability among patients may be underpinned by variations in the immunological mechanisms leading to the loss of tolerance towards CASPR2 (Figure V). In support of this idea, we observed that the patients with a malignant thymoma (who all had Morvan syndrome) usually did not have the anti-CASPR2 autoimmunity-related HLA DRB1*1101 allele, by contrast with the non-paraneoplastic patients (most of whom had limbic encephalitis and/or acquired neuromyotonia) suggesting at least two immunological mechanisms leading to anti-CASPR2 autoimmunity, one that would depend on skewed HLA-dependent antigen presentation, and the other on impaired thymocyte maturation. In addition to the mechanisms of immunization, since some patients with autoimmune episodic ataxia have rare variants of ion channels coding genes, we can speculate that the genetic background of the patient can influence how the nervous system compensates the effects of the antibodies, thereby modulating the symptoms. Therefore, more comprehensive studies of the genome of

CASPR2-Abs patients, especially focusing on VGKC and on other ion channels, may reveal correlations with clinical presentations or specific symptoms and lead to the identification of genetic variants causing functional alterations in ion channels.

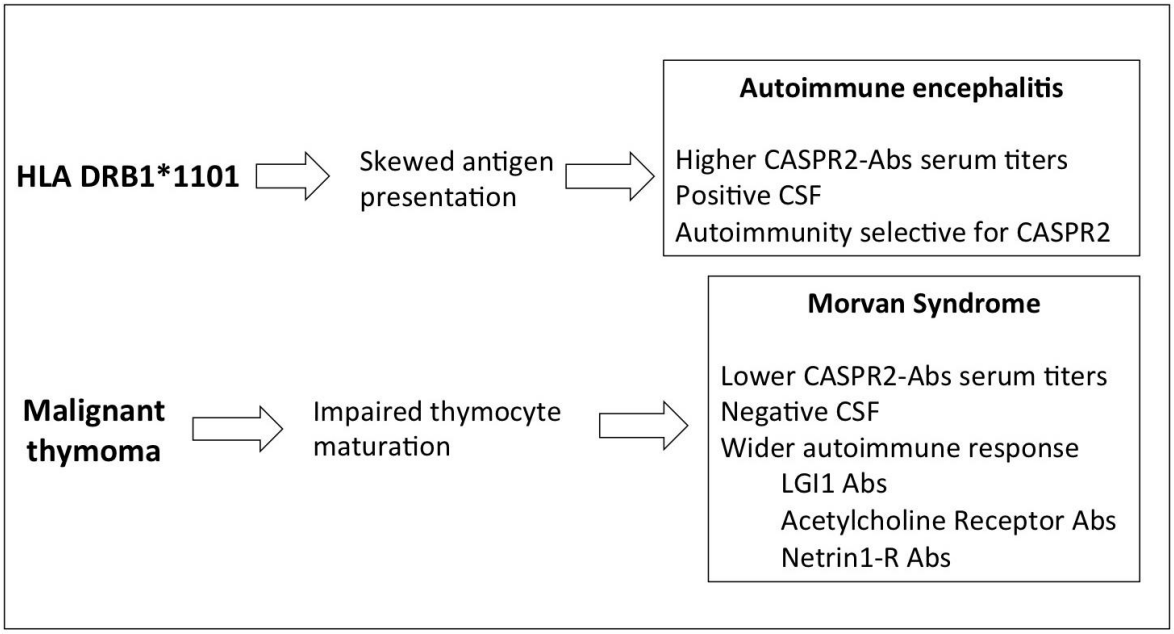

Figure V. Proposed immunological mechanisms accounting for clinical variability. Autoimmune limbic encephalitis patients have distinctive immunological features compared to Morvan syndrome patients. In addition, limbic encephalitis correlates with HLA DRB1*1101 positivity, while Morvan syndrome correlates with malignant thymoma. Therefore, Morvan syndrome and limbic encephaltis likely result from different types of immunological alteration.

Overall, our data hints at the possibility that the neurological phenotype in the patients is not related only to the effect of CASPR2-Abs, but to the entangled consequences of autoantibody effects, autoimmunization mechanisms, and the genetic background of the patients. To verify this hypothesis would imply to study non antibody-mediated mechanisms (such as the role of cellular immunity), or to look after additional antibodies targeting others antigens. It also emphasizes the importance of using samples from carefully selected patients when studying the pathogenic effect of CASPR2-Abs.

STUDYING THE EFFECTS OF CASPR2-ABS: PROJECT UNDERWAY

Since CASPR2-Abs target a neuron surface antigen with possible involvement in synaptic transmission and/or neuronal excitability, and has been shown to disrupt the interaction between CASPR2 and its partner TAG-1, it is likely that CASPR2-Abs have direct, pathogenic effects in the patients (Patterson, Dalmau, and Lancaster 2018). As mentioned above, these effects are incompletely understood, and studies using animal models so far have provided inconclusive results (Dawes et al. 2018; Giannoccaro et al. 2019). Therefore, studying the pathogenic effects of CASPR2-Abs using adequate animal models will be important to decipher the pathophysiology of CASPR2-Abs syndromes. We are currently developing such animal model studies as part of a research fellowship that I am actually carrying out at the Neuroimmunology Program (Pr. J Dalmau, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain). We use animal models to evaluate whether the patients' symptoms are antibody-mediated, and which molecular alterations underlie them. Since the anti-CASPR2 syndromes encompass central nervous system and peripheral nervous system disorders, we are using two different animal models in order to study separately central nervous system and peripheral nervous system affectation: on the one hand, we use a model of antibody-mediated autoimmune encephalitis, in which patients antibodies are chronically infused in the cerebral lateral ventricles of live mice with implanted osmotic pumps; and on the other hand, a model of neuromyotonia, in which patients' antibodies are injected intraperitoneally.

Autoimmune encephalitis model.

We purified serum immunoglobulin G from 6 autoimmune encephalitis patients with CASPR2-Abs (Patients IgGs), and from 6 healthy subjects (Control IgGs) and pooled them at a normalized concentration of 1 μg/μl. The IgGs are infused in the cerebral ventricles of male

C57/Bl6 mice (8-10 weeks old) using osmotic pumps as described elsewhere (Planagumà et al. 2015). For pump implantation, mice are anesthetized and placed in a stereotactic frame and a bilateral catheter will be inserted into the lateral ventricles and secured with dental cement (Figure VI). Each arm of the catheter is connected to one osmotic pump subcutaneously implanted in the back of the mice. For behavioral test, mice are habituated to the experimental room one week before starting the tests, which are performed during the light phase. We assess memory (novel object recognition in open field,), anhedonic behaviour (sucrose preference test), locomotor activity (horizontal and vertical activity assessment, rotarod, footprint analysis), and nociceptive thresholds (Von Frey and Hargreaves tests) at various time points (Figure) (Porsolt, Bertin, and Jalfre 1977; Crawley and Goodwin 1980; Hargreaves et al. 1988; König et al. 1996; Bourquin et al. 2006; Strekalova et al. 2006; Taglialatela et al. 2009; Ennaceur 2010; Guyenet et al. 2010). Animals are sacrificed at determined time points from surgery (3, 18, and 28 days; intracardiac perfusion of a 0.9% saline solution) and brains are retrieved. Brains are fixed one hour in PFA 4%, dehydrated at least 24 hours in sucrose 40%, and then snap frozen in 2-methylbutane chilled in liquid nitrogen. In fixed tissue, determination of human antibodies bound to brain tissue is made using immunoperoxidase staining. Because CASPR2 has been found to regulate Kv1 expression and to be involved in GluA1 synaptic turnover, we study the effects of patients' antibodies on AMPAR and Kv1.1 clusters (Varea et al. 2015; Fernandes et al. 2019; Dawes et al. 2018). To do so, we block non-permeabilized brain sections and incubate them with commercial antibodies against Kv1.1 or the AMPAR subunit GluA1, followed by the corresponding secondary antibodies. We stain synapses using commercial antibodies against the post-synaptic density marker PSD-95 or the pre-synaptic marker Bassoon, after permeabilization. Results are scanned with a confocal microscope in determined hippocampal area (CA1, CA3 and dentate gyrus) to calculate the mean cluster densities for Kv1.1 and

GluA1. The relative densities of synaptic and extrasynaptic Kv1.1 and GluA1 will be estimated studying colocalization of GluA1 with PSD-95 and Kv1.1 with Bassoon, respectively.

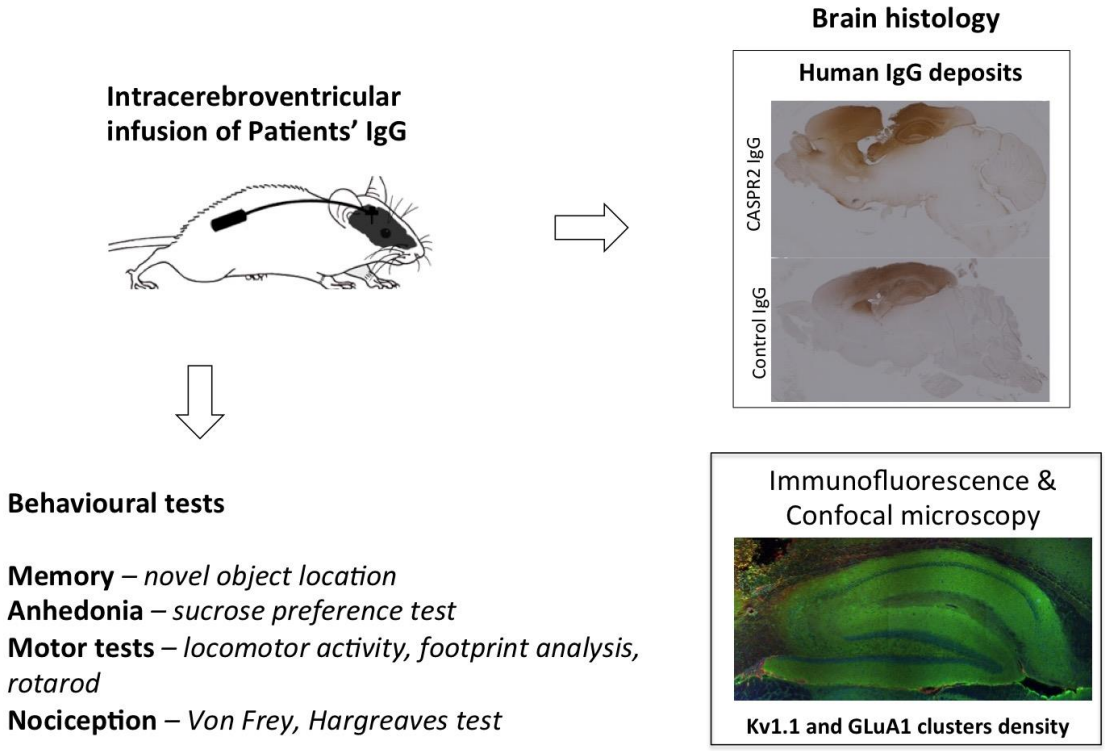

Figure VI. Outlines of the murine model of CASPR2-Abs autoimmune limbic encephalitis.
Purified serum IgGs from CASPR2-Abs positive patients or healthy controls are perfused in the lateral cerebral ventricles of live mice using osmotic pumps. Mice are repeatedly tested over one month for cognitive and motor and nociceptive functions. After one month, the animal are sacrificed and Kv1 and GLuA1 cluster densities are assessed.

Neuromyotonia model.

In parallel to the autoimmune encephalitis model, we use additional batches of mice in order to study the peripheral aspects of the disease, using the neurogenic pain observed in neuromyotonia patients as a clinical indicator of peripheral nerve hyperexcitabiliy. The effects of CASPR2-Abs on nociceptive thresholds will be then correlated to microstructural and molecular alterations in the sciatic nerves and dorsal root ganglia. We inject pooled purified

IgG from 2 neuromyotonia patients with CASPR2-Abs (Patients IgGs) and 4 healthy subjects (Control IgGs) intraperitoneally in mice (5mg/day for 4 days). We assess nociceptive thresholds using the Hargreaves and Von Frey tests at baseline and once a week until day 31 (Bourquin et al. 2006; Hargreaves et al. 1988). At day 32, the animals are anesthetized and perfused intracardiacally with PFA 4%. We collect the cervical spinal cord, dorsal root ganglia, sciatic nerves and glabrous skin from hindpaws. Tissue samples are then fixed in PFA4%, dehydrated and snap frozen as described above. We will then determine the cluster densities of CASPR2 and Kv1 in dorsal root ganglia and sciatic nerve and assess the nodal organization in the sciatic nerves using anti-NaV, anti-CASPR2 and anti-Kv1.2 immunostainings. Human IgGs deposits will be measured in the medulla, dorsal root ganglia, and sciatic nerve. Finally, we will assess intra-epidermal nerve fiber density in the glabrous skin using a PGP9.5 immunostaining.

Expected Results.

This project will allow us 1) to confirm if the symptoms in CASPR2-Abs patients are antibody-mediated both at the central nervous system and peripheral nervous system levels; 2) to study in vivo the potential impact of CASPR2-Abs on glutamatergic synapses, on the expression of Kv1.1 in the brain and in the peripheral nervous system, and on the microstructural organization of peripheral myelinated fibers.

CONCLUSIONS

With this PhD project, we clarify the clinical spectrum associated with CASPR2-Abs and improve the clinical description of the patients with CASPR2-Abs related neurological syndromes. We also pave the way to future animal models able to understand the potential effect of anti-CASPR2 autoantibodies. Importantly, our findings support the classification of CASPR2-Abs patients into three distinct syndromes, which can have important consequences on the management of the patients. In particular, improving the clinical classification of CASPR2 disease is likely to make clinicians more aware of these syndromes, hopefully reducing delays to diagnosis and to treatment. In addition, a better knowledge of these syndromes is an important basis for future clinical studies focusing on the outcomes and on improving the therapeutic strategies in these patients.

Furthermore, our data provide a basis for basic research studies aiming at identifying the molecular disruptions that lead to the neurological symptoms in CASPR2-Abs patients. It is likely that such mechanisms vary according to syndromes, which makes good clinical classification a prerequisite for future basic research studies. In addition, our data suggest that depending on the syndrome, distinct immunological processes lead to the production of CASPR2-Abs. Understanding these processes might prove useful for future clinical or therapeutic studies, and may explain the clinical diversity of CASPR2-Abs patients.

In conclusion, our work contributes to improving our knowledge of CASPR2-Abs syndromes. It brings new paths for clinical and basic research studies, some of which that are underway.

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ANNEXES

List of publications.

Under Review: Muñiz-Castrillo S, Joubert, B, Elsensohn M, et al. Clinical phenotypes in CASPR2-antibody diseases correlate with distinct HLA associations and immunological features. **(THIRD STUDY)**

1. Joubert B, Saint-Martin M, Noraz N, et al. Characterization of a Subtype of Autoimmune Encephalitis With Anti-Contactin-Associated Protein-like 2 Antibodies in the Cerebrospinal Fluid, Prominent Limbic Symptoms, and Seizures. *JAMA Neurol*. 2016;73(9):1115-1124. **(FIRST STUDY)**

2. Joubert B, Gobert F, Thomas L, et al. Autoimmune episodic ataxia in patients with anti-CASPR2 antibody-associated encephalitis. *Neurol Neuroimmunol Neuroinflammation*. 2017;4(4):e371. **(SECOND STUDY)**

3. Torres-Vega E, Mancheño N, Cebrián-Silla A, et al. Netrin1-receptor and Caspr2 antibodies predict thymoma in myasthenic patients with neuromyotonia. *Neurology*. **(ANNEX 1)**

4. Saint-Martin M, Joubert B, Pellier-Monnin V, Pascual O, Noraz N, Honnorat J. Contactin-associated protein-like 2 (CASPR2), a protein of the neurexin family involved in several human diseases. *Eur J Neurosci*. July 2018. **(ANNEX 2)**