CLINICAL AND LABORATORY DIAGNOSTIC ASSESSMENT OF IMMUNE-MEDIATED NEUROLOGICAL DISORDERS

Ph.D. Thesis

Zsófia Hayden M.D.

Department of Immunology and Biotechnology

Medical School

University of Pécs, Hungary

Leaders of the project: Prof. Timea Berki, M.D., Ph.D., Ds.C.

Beáta Bóné, M.D., Ph.D.

Leader of the program: Prof. Timea Berki, M.D., Ph.D., Ds.C

Leader of Doctoral School: Prof. Dóra Reglődi, M.D., Ph.D., Ds.C.

Pécs, 2021

TABLE OF CONTENTS

Dissertation Guidance

The dissertation provides information about three antibody-mediated neurological disorders: 1) onconeuronal antibody-associated syndromes, 2) neuronal cell surface-antibody mediated autoimmune encephalitis, and 3) neuromyelitis optica spectrum disorder. All data summarized within each chapter has been published as individual projects on its own right. The thesis involves three independent studies centred around pathological and natural autoantibodies. The dissertation discusses laboratory and clinical characteristics of antibody-mediated neurological disorders and B cell activation in autoimmune CNS disorders resulting in pathological and natural autoantibody production.

List of abbreviations

AE: autoimmune encephalitis

AMPAR1/2: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor ½

ANNA-1: type 1 antineuronal nuclear antibodies

ANNA-2: type 2 antineuronal nuclear antibodies

APC: antigen-presenting cells

AQP4: aquaporin-4

BBB: blood-brain barrier

Caspr2: contactin-associated protein-like 2

CNS: central nervous system

CSF: cerebrospinal fluid

CTLs: $CD8^+$ cytotoxic T lymphocytes

CV2/CRMP5: collapsin response mediator protein 5

dcSSC: diffuse cutaneous systemic sclerosis

DLBCL: diffuse large B-cell lymphoma

DMT: disease modifying therapy

DN: double negative

DPPX: dipeptidyl-peptidase-like protein-6

dsDNA: anti-double stranded DNA

EAE: experimental autoimmune encephalomyelitis

EBV: Epstein–Barr virus

EDSS: Expanded Disability Status Scale

EEG: electroencephalography

FBDS: faciobrachial dystonic seizures

FLAIR: fluid-attenuated inversion recovery

GABAAR: γ-aminobutyric acid receptor-A

GABABR: γ-aminobutyric acid receptor-B

GAD65: glutamic acid decarboxylase (65kDa)

GM-CSF: granulocyte-macrophage colony-stimulating factor

HCs: healthy controls

HEK293: human embryonic kidney 293

HL: Hodgkin lymphoma

HSV: herpes simplex virus

IFN-γ: interferon-gamma

Ig: immunoglobulin

IL: interleukin

IPND: International Panel of NMO Diagnosis

IQR: interquartile ranges

IVIG: intravenous immunoglobulin

LCNEC: large cell neuroendocrine carcinoma

LE: limbic encephalitis

LGI1: leucine-rich, glioma inactivated 1

MHC: major histocompatibility complex

MOG: myelin oligodendrocyte glycoprotein

MS: multiple sclerosis

MRI: structural magnetic resonance imaging

mRS: modified Rankin scale

nAbs: natural autoantibodies

NHL: non-Hodgkin lymphoma

NMDAR: N-methyl-D-aspartate receptor

NMO: neuromyelitis optica

NMOSD: neuromyelitis optica spectrum disorder

NS: non-switched

NSCLC: non-small-cell lung carcinoma

OCB: oligoclonal bands

PAMPs: pathogen-associated molecular patterns

PBMCs: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PCA-1: purkinje cell cytoplasmic antibody type 1

PCD: paraneoplastic cerebellar degeneration

PNS: peripheral nervous system

PNSs: paraneoplastic neurologic syndromes

PRRs: pattern-recognition receptors

qPCR: quantitative Polymerase Chain Reaction

RP105: radioprotective 105 kDa

RRMS: relapsing-remitting multiple sclerosis

RQ: relative quantification

S: switched

SCLC: small cell lung cancer

SEM: standard error of the mean

SLE: systemic lupus erythematosus

SOX1: sry-like high mobility group box protein 1

SPMS: secondary progressive multiple sclerosis

TGF-β: transforming growth factor beta

TIR: Toll-IL-1R

TMB: 3,3',5,5'-tetramethylbenzidine

TNF-α: tumour necrosis factor alpha

TLRs: Toll-like receptors

Tr/DNER: delta/notch-like epidermal growth factor-related receptor

Zic4: zinc finger protein 4

VGKC: voltage-gated potassium channel complex

1. INTRODUCTION

1.1 General overview of onconeuronal antibody-associated syndromes, neuronal cell surface-antibody mediated autoimmune encephalitis and neuromyelitis optica spectrum disorder

1.1.1 Comparison of onconeuronal antibody-associated syndromes and neuronal cell surface-antibody mediated autoimmune encephalitis

From 1980 until 2000, many classical intracellular onconeuronal antibody-associated syndromes, defined as paraneoplastic neurologic syndromes (PNSs) have been reported, including anti-Yo, anti-Hu, anti-amphiphysin, anti-CV2/CRMP5 (collapsin response mediator protein 5) and anti-Ma autoantibodies. Since 2007, the definition of autoimmune encephalitis (AE) was introduced by the discovery of neuronal cell surface or synaptic autoantibodies, such as anti-NMDAR (N-methyl-D-aspartate receptor), anti-AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor), anti-LGI1 (leucine-rich, glioma inactivated 1), anti-Caspr2 (contactin-associated protein-like 2), and anti-GABABR (γ-aminobutyric acid receptor-B) autoantibodies.

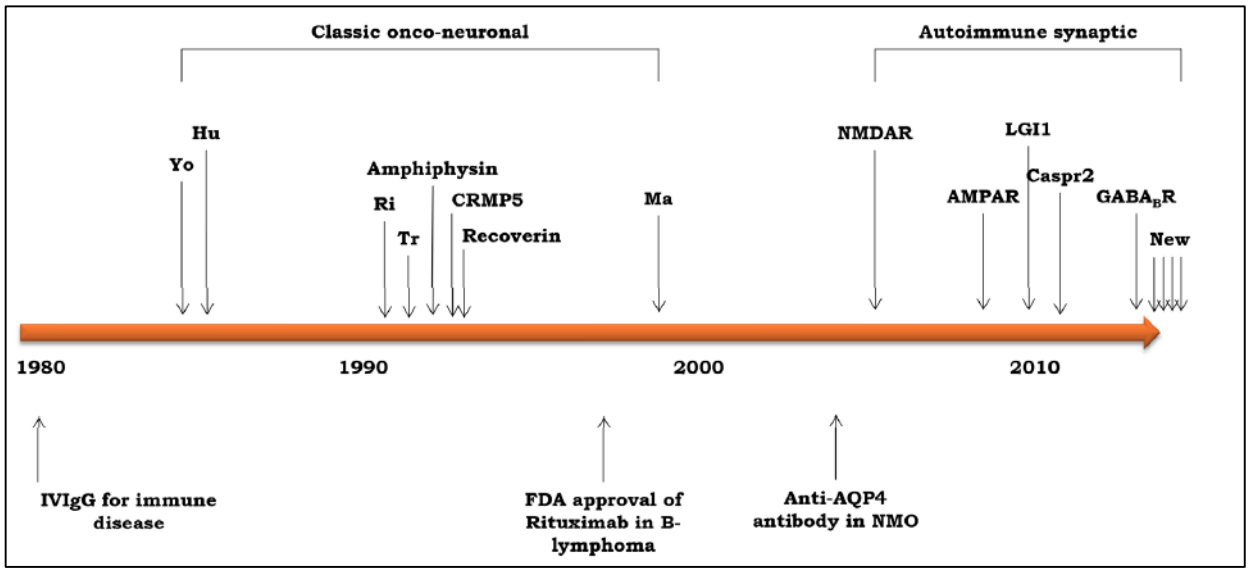


Figure 1. The discovery of onconeural and neuronal cell surface autoantibody-associated neurological syndromes. *Adapted from Lee et al. 2016 (1).*

The distinction between PNSs associated with autoantibodies against intracellular neuronal antigens and neuronal cell surface antibody-mediated AE is crucial. In spite of some similar triggers and neurological syndromes, prevalence of associated systemic tumours,

pathogenic mechanisms and outcomes differ between these disease groups (2). In onconeural antibody-associated PNSs, systemic tumour association is frequent, occurring in >90% of cases, whereas, neuronal cell surface antibody-mediated AE is variably associated with tumours.

Table 1. Characteristics of CNS syndromes associated with onconeural and neuronal cell surface antibodies. *Based on Zuliani et al. 2012 (3).*

	Classical paraneoplastic CNS syndromes associated with onconeural antibodies	**CNS syndromes associated with neuronal surface antibodies**
Antibodies	Antibodies against intracellular antigens (Hu, Yo, Ri, Ma2, Cv2/CRMP5, amphiphysin, Sox1/2)	Antibodies against neuronal cell surface receptors (NMDAR, GABABR, AMPAR1, AMPAR2), or associated proteins (LGI1, Caspr2)
Pathogenic mechanism	T cell cytotoxicity is the proposed mechanism; antibodies are not likely to be pathogenic, only markers for the tumour	Autoantibodies are pathogenic (downregulation of target antigen /complement mediated damage)
Age (range; years) and sex	Mainly adults (40–70); both genders (PCD more frequent in women)	Occur in persons of all ages (anti-NMDAR encephalitis common in children and young women)
Tumours	Common SCLC, breast, ovary, testicular	No tumour found in many cases (particularly in anti-LGI1 encephalitis) Teratoma, thymoma, SCLC, breast
Prognosis	Poor; improvement or stabilisation related mainly to tumour treatment	Variable but generally good; possible spontaneous remission
Immunotherapy	Not usually effective	Generally effective

The better outcome of AE might be explained by reversible neuronal dysfunction, which is caused by pathologic autoantibodies binding to extracellular epitopes of neuronal cell surface proteins, and altering their structure and function. In spite of AE, in PNSs with autoantibodies against intracellular proteins, the antibodies are not likely to be pathogenic as they cannot reach the intracellular epitopes, instead cytotoxic T cells are involved, leading to irreversible neuronal loss, which may explain the poor prognosis in these patients. In AE, immunotherapy (e.g. steroids, plasmapheresis, immunosuppression, IVIG) results in the reduction of autoantibody levels and can lead to the improvement of patients. Conversely, in PNSs, T cell-mediated irreversible neuronal damage is frequently present, thus immunotherapies are generally not effective; however, proper treatment of the tumour can lead to stabilization of the patients.

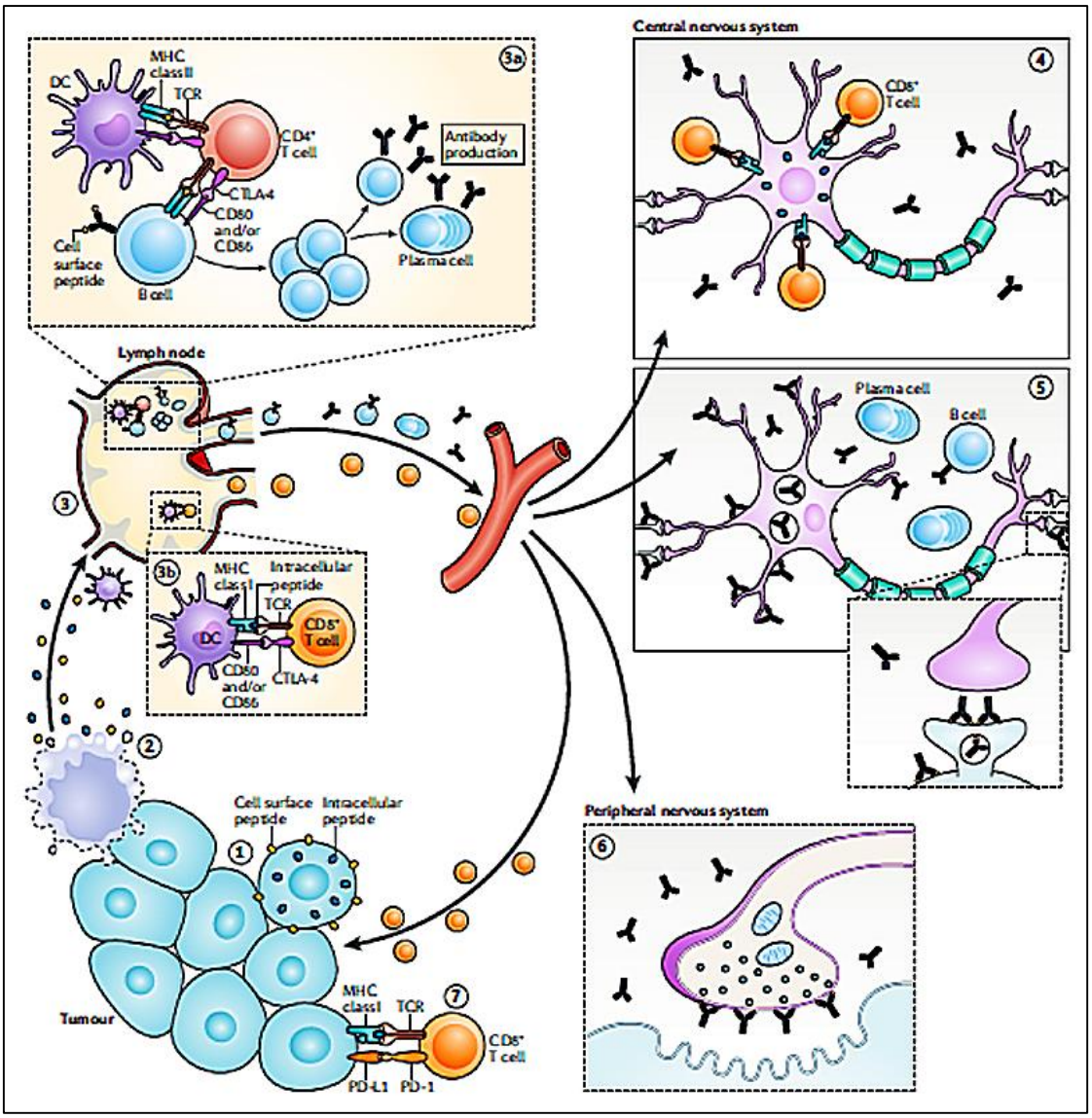


Figure 2. Proposed mechanisms of CNS syndromes associated with onconeural and neuronal cell surface antibodies, in this case the disease is triggered by a systemic tumour. The blue ovoid shapes indicate intracellular antigens and the yellow ovoid shapes show neuronal cell surface receptors or proteins. Antigens are expressed in the tumour (step 1) and are released upon apoptosis of the tumour cell (step 2). Proteins are taken up and processed by dendritic cells and presented within the regional lymph nodes to the cells of the adaptive immune system (step 3). CNS syndromes associated with neuronal cell surface antibodies are predominantly mediated by B cells and autoantibodies (humoral immune response) (step 3a), which bind to their target in the brain causing structural and functional alterations (step 5), or reach target directly in the PNS (step 6). In contrast, CNS syndromes associated with intracellular antibodies are mediated by $CD8^+$ cytotoxic T lymphocytes (cellular immune response) (step 3b), causing irreversible neuronal loss (step 4). *Adapted from Graus et al. 2019 (4).*

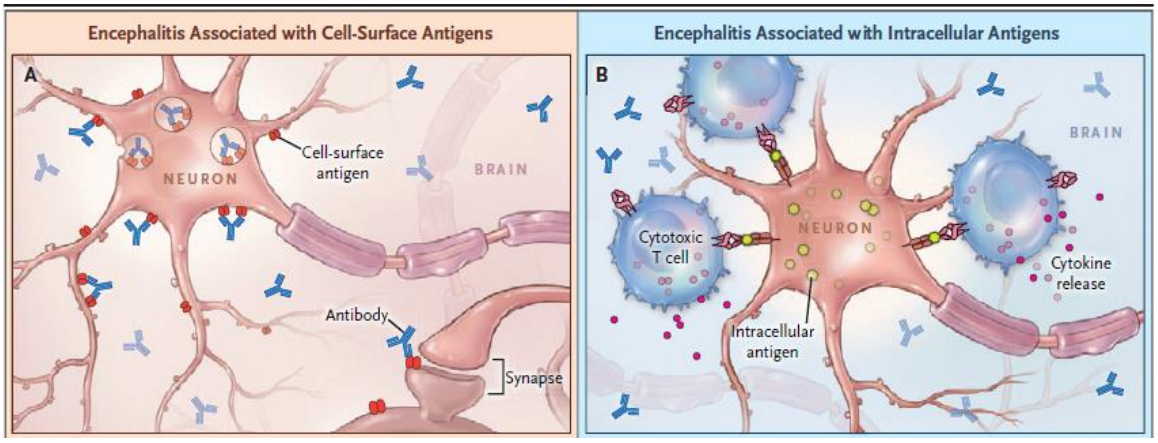


Figure 3. Neuronal cell surface antibody-mediated autoimmune encephalitis is characterized by reversible neuronal dysfunction caused by pathogenic autoantibodies (Panel A), whereas, paraneoplastic neurologic syndromes associated with intracellular neuronal antigens is characterized by cytotoxic T cell-mediated irreversible neuronal dysfunction (Panel B). *Adapted from Dalmau et al. 2018 (2).*

Besides clinical features and auxiliary examinations, such as electroencephalography (EEG) and structural magnetic resonance imaging (MRI), accurate diagnosis of PNSs and AE requires detection of characteristic autoantibodies in the serum and/or cerebrospinal fluid (CSF). Diagnosis especially may be complicated in AE, due to the often unremarkable cerebral imaging and CSF analysis results, especially in the elderly with anti-LGI1 and anti-Caspr2 encephalitis (5). This highlights the importance of early clinical diagnosis of these disease groups, in which the laboratory has a crucial role by accurate and reproducible testing of serum and/or CSF samples for the presence of autoantibodies.

1.1.2 Definition, incidence, mechanism and diagnosis of onconeuronal antibody-associated syndromes

Paraneoplastic neurologic syndromes (PNSs) comprise a heterogeneous group of disorders associated with cancer that can affect any part of the nervous system (central, peripheral and/or autonomic) and can manifest in the form of focal (eg. cerebellar degeneration) or general (eg. encephalomyelitis) neurological disorders (6). The disease was first decribed by the French physician Auche` in the 19th century, who linked peripheral nervous system (PNS) involvement to remote neoplasm effects in patients diagnosed with cancer (7). In the mid-20th century, the term ‘paraneoplastic neurologic syndromes’ was introduced to encompass neurological disorders associated with cancer and not caused by direct tumour invasion, metastasis, side

effects of oncological treatment, metabolic and nutritional deficits, infections or coagulopathy (8-10). The concept of immune-mediated pathogenesis was raised in 1951 and was supported by the presence of 'onconeural antibodies' in patient's sera with neurological symptoms and tumour. PCA-1 (Purkinje cell cytoplasmic antibody type 1) was the first antibody type described in patients with paraneoplastic cerebellar degeneration (PCD) and ovarian carcinoma in 1983 (11). Since then, multiple autoantibodies and related syndromes have been described and the list of possible target antigens increases continuously.

PNSs are rare disorders, estimates show that 0.5-1% of patients with cancer have clinically disabling PNSs, although convincing epidemiologic data are missing. Prevalence varies based on the type of associated tumour, and can occur in 2–3% of patients with neuroblastoma or small cell lung carcinoma (SCLC), whereas, 30–50% of patients with thymoma and sclerotic myeloma present with PNSs (7).

The immune pathogenesis has been confirmed in case of some PNSs, the best-known hypothesis claims that disease progression is driven by autoimmune responses based on molecular mimicry. Ectopic expression of neuronal proteins by malignant neoplasms leads to the activation of the immune system and further onconeural autoantibody production against intracellular proteins of the nervous system, such as Hu, Yo, Ri, CV2, Ma2, amphiphysin, Tr, GAD65, Zic4, titin, SOX1, recoverin. In PNSs, the tumour expresses intracellular antigens that are released upon apoptosis. These proteins are taken up and transferred to the regional lymph nodes by dendritic cells, where they are presented to the $CD4^+$ helper T lymphocytes. The activated $CD4^+$ helper T lymphocytes cross-react with neuronal antigens in the CNS and lead to inflammation, and further activate B cells to produce antibodies against tumour antigens. The $CD8^+$ cytotoxic T lymphocytes (CTLs) are activated by T cell receptor-mediated recognition of the antigen, in the context of MHC class I molecules expressed on the surface of dendritic cells. The CTLs lead to irreversible neuronal damage by entering the central nervous system (4).

Although, PNSs can manifest in the form of diverse neurological symptoms, there are some common features that apply to most of the syndromes: 1) symptoms precede tumour detection in 70% of patients, 2) disease onset is usually subacute, 3) presence of onconeural autoantibodies are highly predictive for an underlying tumour, and 4) autoantibody characterization can aid adequate tumour screening.

The disease is often accompanied by autoantibody production against intracellular antigens, although they are not likely to be pathogenic, rather T cell-mediated responses against the target antigens might be responsible for disease development (12). However, these autoantibodies may serve as biomarkers of PNSs and onconeural autoantibody detection in sera can almost always indicate the presence of an underlying neoplasm. Characterization of the associated antibodies is important as each onconeural autoantibody is associated with different types of cancer, making it possible to conduct targeted tumour screening in affected patients, and also raising the possibility of the presence of a second, occult malignancy, if the underlying tumour has a different histological type than expected. The fact that response to immunosuppressive therapy is also different based on the cellular location of the target antigen, also emphasizes the role of autoantibody detection in the diagnosis of these clinical syndromes.

Diagnostic criteria for PNSs is based on the presence or absence of tumour, and the definitions of classical syndrome and well characterized onconeural antibodies (Table 2). Classical neurological syndromes are often associated with tumour, and includes symptoms affecting the CNS, such as encephalomyelitis, limbic encephalitis (LE), subacute cerebellar degeneration, opsoclonus-myoclonus, and symptoms affecting the PNS, like subacute sensory neuronopathy, chronic gastrointestinal pseudo-obstruction, and also symptoms affecting the neuromuscular junction or the muscles, such as Lambert-Eaton myasthenic syndrome and dermatomyositis.

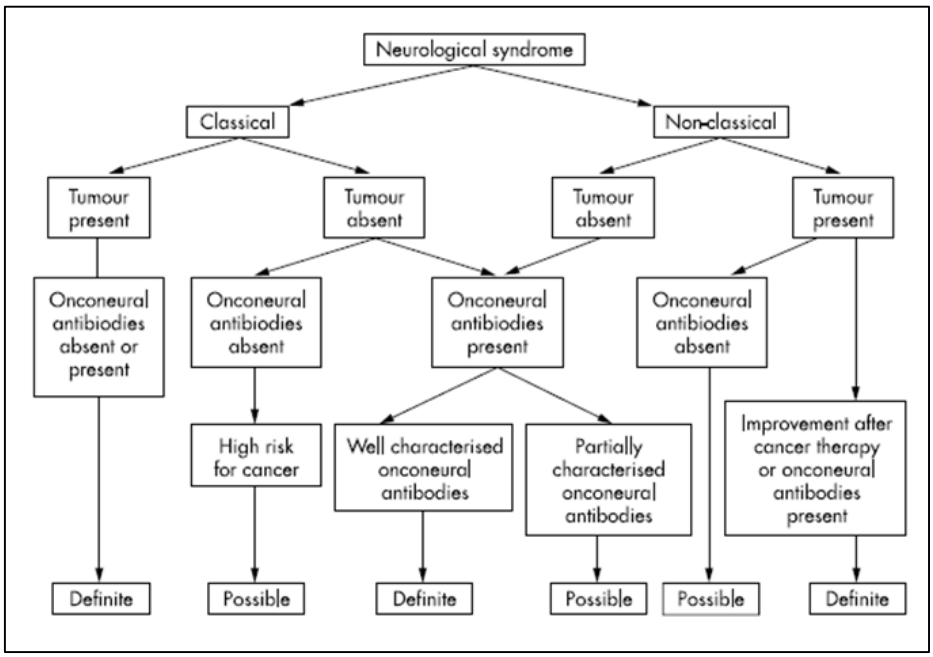


Figure 4. Based on the recommendations by Graus et al. 2009 (13), PNSs can be defined as definite or possible.

Table 2. Characteristics of onconeural autoantibody-associated PNSs. *Based on (7, 14-21).*

Autoantibody	Age (median; years) and sex predominance	Clinical features	Tumour association	Prognosis
Well characterized onconeural antibodies				
Hu (ANNA-1)	63 years; Females	Encephalomyelitis (cortical, limbic, brainstem encephalitis), cerebellar degeneration, myelitis, sensory neuropathy, autonomic dysfunction	>90%; SCLC, NSCLC, extra-thoracic cancers	Poor response to treatment
Yo (PCA-1)	60 years; Females	Cerebellar degeneration	>90%; Ovary, breast, fallopian tube	Poor response to treatment
CV2/CRMP5	68 years; Males	Encephalomyelitis, cerebellar degeneration, chorea, peripheral neuropathy, uveitis	>90%; SCLC, NSCLC, thymoma, extra-thoracic cancers	Poor response to treatment
Ri (ANNA-2)	66 years; Females	Cerebellar degeneration, brainstem encephalitis, opsoclonus-myoclonus	>85%; Breast, ovary, SCLC	Poor response to treatment
Ma2	55 years; Males	Limbic, brainstem, hypothalamic encephalitis, rare: cerebellar degeneration	>90%; Men <45 years: testicular germ cell tumours	One-third of young men may improve with treatment, older patients rarely improve
Amphiphysin	72 years; Males	Stiff person syndrome, encephalomyelitis, limbic encephalitis, myelopathy	>80%; SCLC, breast	Often improves with treatment
Partially characterized onconeural antibodies				
Tr/DNER	61 years; Males	Cerebellar degeneration	>90%; HL	80% are men <45 years, about 20% of patients respond to treatment
Recoverin	55 years; Females	Retinopathy	SCLC	Poor response to treatment
Zic4	67 years; Males	Cerebellar degeneration	92%; HL	Poor response to treatment
Titin	56 years, Males	Myasthenia gravis	Thymoma	Poor response to treatment
SOX1	72 years; Males	Lambert-Eaton myasthenic syndrome, neuropathy	>95%; SCLC, NSCLC, extra-thoracic cancers	Poor response to treatment
GAD65	40 years; Females	LE, stiff person syndrome, cerebellar ataxia, opsoclonus-myoclonus	18%; SCLC, neuroendocrine, thymoma, breast, NHL	Limited response

1.1.3 Definition, incidence and mechanism of neuronal cell surface-antibody mediated AE

During the past few years it has been recognized that there are CNS disorders presenting in the form of LE, in which the presence of autoantibodies against the neuronal cell surface receptors, such as NMDAR, GABABR, and AMPAR, or synaptic proteins associated with the VGKC (voltage-gated potassium channel complex), such as LGI1 and Caspr2, has been documented, and shown to be responsible for the development of the symptoms (2, 22, 23). The neuronal synapses receptor proteins, which are the target molecules of the autoantibodies, play important roles in synaptic signal transmission and neuronal plasticity (22). The autoimmune reaction to these antigens in the majority of cases leads to prominent neuropsychiatric symptoms and epileptic seizures (24-28). AE can occur in persons of all ages, although, some AE subtypes affect predominantly children and young adults. Patients can have a fatal outcome due to the lack of proper therapy. This highlights the importance of early clinical diagnosis of AE, in which the laboratory has a crucial role by providing accurate and reproducible testing of serum and/or CSF samples for the presence of autoantibodies.

The annual incidence of encephalitis is approximately 5-8/100,000/year, and in almost half of the cases, the exact cause cannot be established (29). Data regarding the prevalence and incidence of AE subtypes are limited. A prospective, multicenter study described that following viral infections and acute disseminated encephalomyelitis, autoimmune etiologies are the third most common cause of encephalitis (29). The California Encephalitis Project (30) reported of higher frequency of anti-NMDAR encephalitis, which is considered to be the most common AE subtype, compared to viral encephalitis in younger persons, and it was also described that anti-NMDAR encephalitis accounted for 1% of intensive care admission among young adults (31). A retrospective Dutch study reported of anti-LGI1 encephalitis as the second most frequent AE subtype, following anti-NMDAR encephalitis, with an incidence of 0.83/1,000,000/year (26).

Two potential triggers of AE are systemic tumours and herpes simplex virus (HSV) encephalitis (2). The ectopic expression of neuronal proteins by the tumour cells serve as targets of the autoantibodies, which might result in the initiation of an autoimmune response (32). HSV encephalitis can trigger autoantibody production against NMDAR, occurring in approximately 20% of patients in the form of relapsing neurological symptoms weeks after the onset of HSV encephalitis (33-35). The autoimmune response is initiated by antigens released by viral destruction of neurons or apoptotic tumour cells, and processed and transported to the regional

lymph nodes by antigen-presenting cells (APC; dendritic cells). In the lymph nodes, naïve B cells and $CD4^+$ T cells will be exposed to antigens, and B cells differentiate into antibody-producing plasma cells. Memory B cells enter the brain, where they undergo restimulation, antigen-driven affinity maturation, clonal expansion, and differentiation into antibody-producing plasma cells (2).

In AE, the autoantibodies bind to the extracellular epitopes of the neuronal cell surface receptors or their associated proteins, which can lead to the alteration of the structure and the function of the target antigens by different mechanisms. The pathogenic effects of these antibodies have been shown in primary cultures of neurons. Thus in anti-NMDAR encephalitis, autoantibodies induce receptor cross-linking and internalization (36, 37), in anti-LGI1 encephalitis, autoantibodies interfere with protein-protein interactions, which affect the function of VGKC, and decrease the level of AMPAR (38), and in anti-GABABR encephalitis, autoantibodies may block the function of the target antigen (2). The autoantibodies cause reversible neuronal dysfunction, and immunotherapy, such as steroids, plasmapheresis, immunosuppression, and IVIG, results in reduction of autoantibody levels, and can lead to the improvement of patients (39).

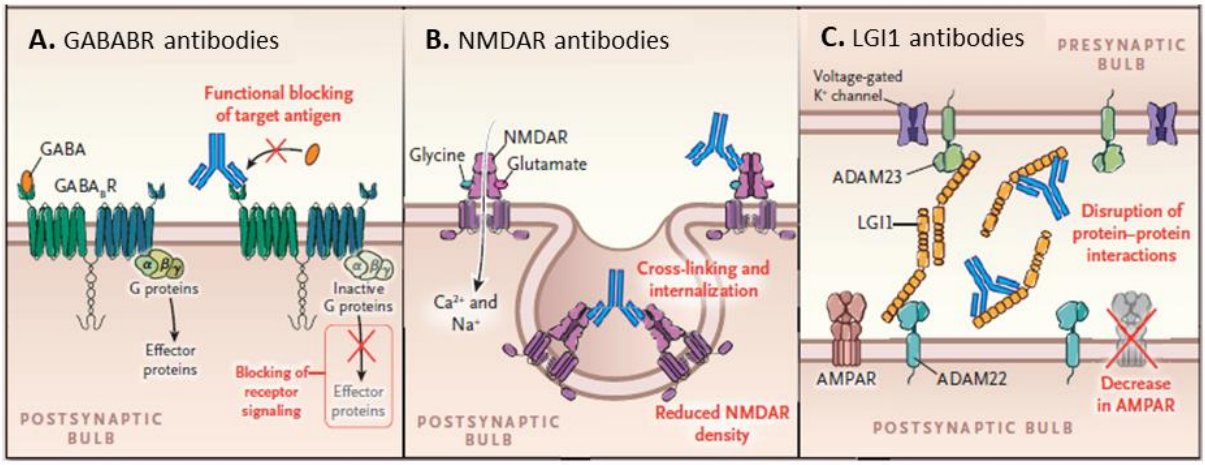


Figure 5. AE-related autoantibodies lead to neuronal dysfunction through various mechanisms, including functional blocking of the target antigen (Panel A, anti-GABABR antibodies), receptor cross-linking and internalization (Panel B, anti-NMDAR antibodies) and disruption of protein-protein interactions, altering the function of VGKC and decrease AMPAR levels (Panel C, anti-LGI1 antibodies). *Modified image adapted from Dalmau et al. 2018 (2).*

Table 3. Characteristics of AE subtypes, including age and sex distribution, clinical symptoms, brain MRI findings, tumour associations and prognosis.

Autoantibody	Predominant IgG class	Male/ Female	Median age (range; years)	Clinical features	Brain MRI (T2/FLAIR)	Tumour association	Prognosis
NMDAR	IgG1	1:4	21 years (2 mo-85 year)	Prodromal stage (fever, headache, abdominal pain) Psychiatric symptoms (agitation, hallucinations, delusions, catatonia, psychosis) Later manifestations (reduction of speech, memory deficit, orofacial and limb dyskinesias, seizures, decreased level of consciousness, autonomic instability)	Normal or non-specific changes	58%, (age and sex dependent) in young women ovarian teratoma	81% have a good outcome
LGI1	IgG4	2:1	64 years (31-84)	Faciobrachial dystonic seizures, limbic encephalitis, hyponatraemia, sleep disorders, memory and cognitive deficits	Hyperintense signal in medial temporal lobes	<5%, thymoma	70% have a good outcome
Caspr2	IgG4	9:1	66 years (25-77)	Neuromyotonia, Morvan's syndrome, limbic encephalitis, insomnia, neuropathic pain	Hyperintense signal in medial temporal lobes	<5%, thymoma	70% have a good outcome
GABABR	IgG1	1.5:1	61 years (16-77)	Limbic encephalitis, seizures Rarely: cerebellar ataxia, opsoclonus-myoclonus	Hyperintense signal in medial temporal lobes	50%, SCLC	80% initially good response but have poor prognosis due to SCLC
AMPAR	IgG1	1:2.3	56 years (23-81)	Limbic encephalitis, seizures Rarely: psychiatric symptoms	Hyperintense signal in medial temporal lobes	56%, SCLC, thymoma or breast carcinoma	70% have a good outcome

1.1.4 Characteristics of neuronal cell surface-antibody mediated AE subtypes

The different AE subtypes may present with a wide spectrum of clinical symptoms, such as behavioral and psychiatric disorders, cognitive impairment, decreased level of consciousness, seizures, movement disorders and dysautonomia (39, 40). This section of the Ph.D. thesis summarizes characteristics of those neuronal surface antibody-mediated AE subtypes, including anti-NMDAR, anti-LGI1, anti-GABABR, anti-Caspr2 and anti-AMPAR encephalitis, for which autoantibody testing is available in the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary. The section also discusses those neuronal surface antibody-mediated AE subtypes, which are associated with recently discovered autoantibodies, including anti-DPPX (dipeptidyl-peptidase-like protein 6), anti-IgLON5 and anti-GABAAR (γ-aminobutyric acid receptor-A) antibodies, for which autoantibody testing is not available in our laboratory up to the present time.

1.1.4.1 Anti-NMDAR encephalitis

Since the discovery of autoantibodies against the GluN1 subunit of the NMDAR in 2007 (41), anti-NMDAR encephalitis is now considered as the most frequent AE subtype, and one of the most common cause of encephalitis (42). 80% of anti-NMDAR patients are female with a median age of onset 21 years (range: 2 months-85 years) (2), however, Titulaer et al. (43) reported of higher proportion of male patients <12 years or >45 years. Approximately 58% of young female patients have an ovarian teratoma, whereas, in children and men, tumour association is less frequent. Approximately 70% of patients present with prodromal symptoms, including fever, flu-like symptoms, headache, vomiting and diarrhea (42, 44). Teenagers and adults often present with psychiatric symptoms, such as agitation, hallucinations, delusions or catatonia, which may result in hospital admission with the misdiagnosis of psychosis. Within days or weeks, with disease progression, other symptoms, including reduction of speech, memory deficit, movement disorders (orofacial and limb dyskinesias), seizures, decreased level of consciousness and autonomic instability, hypoventilation and even coma can develop (45). In approximately 5% of patients, the disease remains monosymptomatic (e.g. isolated psychiatric symptoms, dystonia or seizures) (23).

1.1.4.2 Anti-LGI1 and anti-Caspr2 encephalitis

Although, initial reports suggested that VGKC complex antibodies bind directly to Kv1.1/Kv1.2 potassium channel subunits (46, 47), autoantibodies primarily targeting two proteins associated to the VGKC, including LGI1 and Caspr2 have been described in 2010 (48, 49).

LGI1 is a secreted protein, mainly present in the hippocampus and the temporal cortex, which can bind proteins of the ADAM (a disintegrin and metalloproteinase) family. LGI1 binds to the presynaptic ADAM23 and also to the postsynaptic ADAM22, including the presynaptic potassium channel and the postsynaptic AMPAR, and regulates the AMPAR mediated synaptic signal transmission. Autoantibodies against LGI1 reduce LGI1-ADAM interaction and reversibly reduce postsynaptic AMPAR clusters (38, 50).

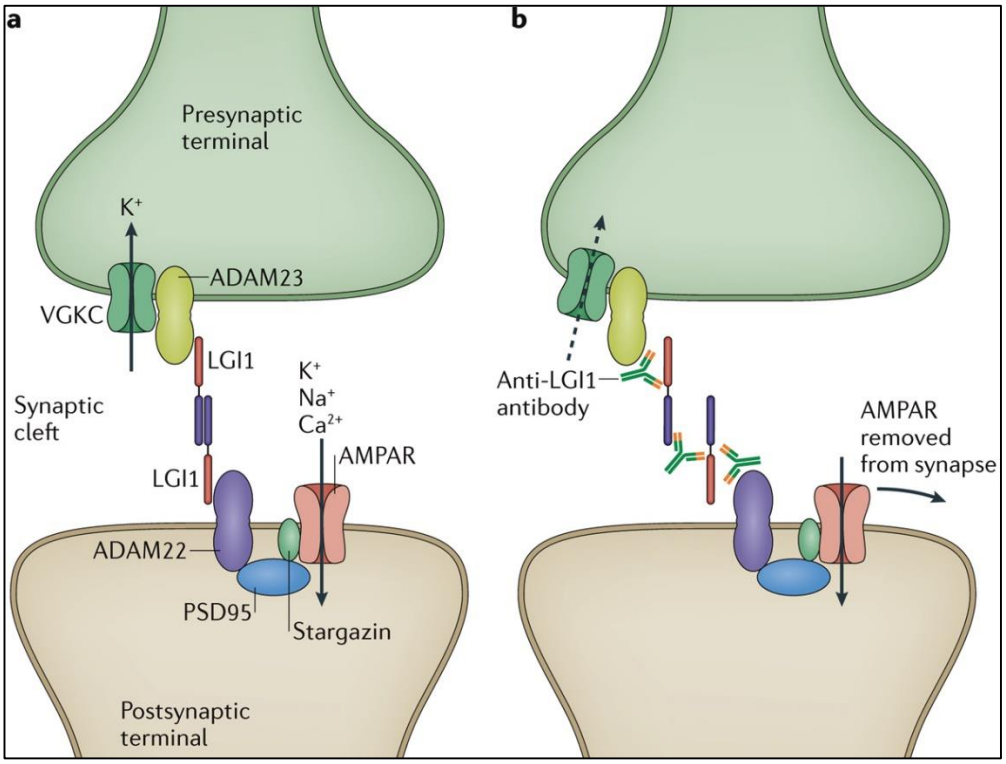


Figure 6. LGI1 connects the presynaptic voltage-gated potassium channel (VGKC) complex and postsynaptic AMPAR through the ADAM22-LGI1-ADAM23 complex (a). Anti-LGI1 autoantibodies bind to LGI1 and alter neuronal excitability (b). *Adapted from van Sonderen et al. 2017 (26).*

LGI1 encephalitis patients account for the majority of cases of LE in the elderly. The syndrome is characterized by male predominance with a median age of onset 64 years (range: 31-84 years). LGI1 encephalitis is usually non-paraneoplastic, an underlying tumour is rare, occurring in <5% of patients (2, 23). Prior to developing the classical symptoms of LE, often a few weeks before onset of cognitive deficit, 26–71% of patients develop faciobrachial dystonic seizures (FBDS) (26). The FBDS is a rare form of epilepsy, which is characterized by frequent brief unilateral dystonic posturing of the upper limb and face, and were recognized as immunotherapy-responsive disorders (51). During disease course, lack of immunotherapy leads to the development of LE, characterized by memory disturbance, confusion, neuropsychiatric symptoms and seizures (48, 49, 52). A characteristic laboratory finding is hyponatremia in 65% of anti-LGI1 encephalitis patients (53). Besides these characteristic features, some rare manifestations, such as piloerection related to anti-LGI1 encephalitis was also described (54).

Caspr2 is a transmembrane axonal protein belonging to the Neurexin IV superfamily, which is expressed both in the CNS and PNS (55). Its cytoplasmic domain has a critical role in potassium channel clustering in the juxtaparanodal region of myelinated axons (56). Antibodies target multiple epitopes of the Caspr2 protein, and react both with brain and peripheral nerves (57), leading to variable syndromes involving the CNS and PNS. Anti-Caspr2 encephalitis almost exclusively affect older males with a median age of onset 66 years (range: 25-77 years), and has a different clinical spectrum compared to anti-LGI1 encephalitis. LE is less frequent, whereas peripheral nerve hyperexcitability syndromes, such as Morvan syndrome are more common (48, 58). Morvan syndrome is a rare constellation of neurological symptoms, characterized by neuromyotonia, pain, hyperhydrosis, weight loss, severe insomnia and hallucinations (59).

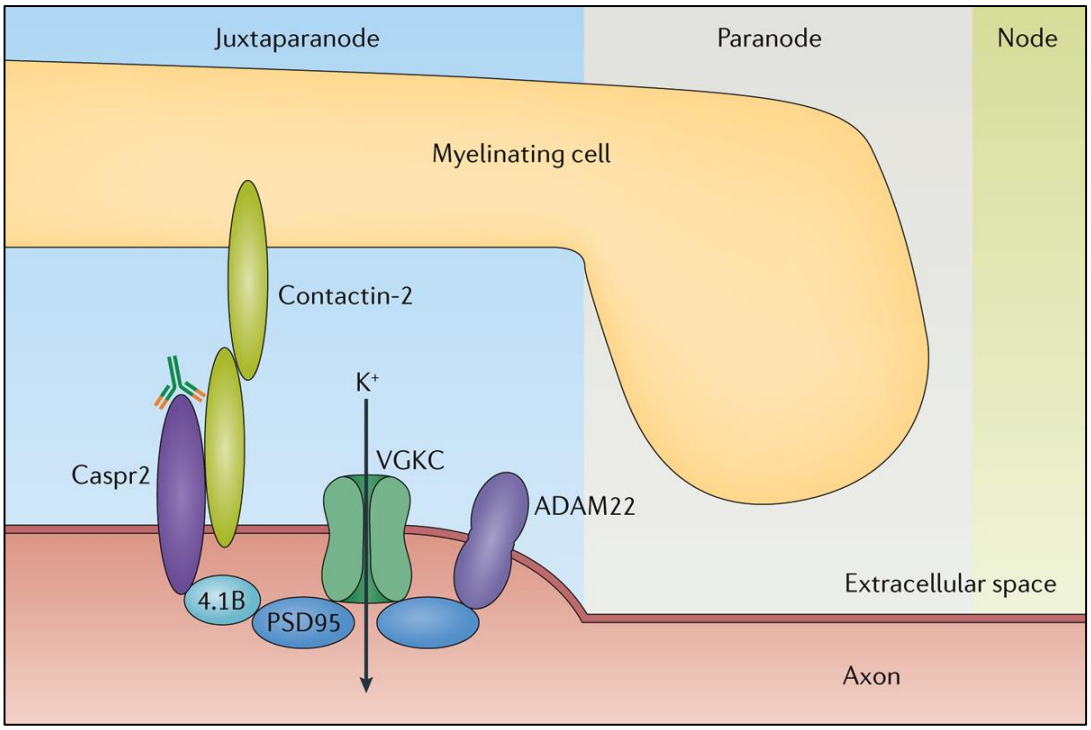


Figure 7. Contactin-associated protein-like 2 (Caspr2) binds to the dimerized contactin-2 and to PSD95. This complex organizes Kv1 potassium channels. Anti-Caspr2 autoantibodies bind to the extracellular region of Caspr2, affecting the Caspr2–contactin-2 interaction. *Adapted from van Sonderen et al. 2017(26).*

1.1.4.3 Anti-GABABR encephalitis

GABABR are G protein-coupled receptors, which are broadly expressed in the nervous system, and play role in affecting neuronal excitability. GABABR have inhibitory action via activating inwardly rectifying K^+ channels, inactivating voltage-gated Ca^{2+} channels, and inhibiting adenylate cyclase (60). Anti-GABABR encephalitis was first described in 2010 by Lancaster et al. (32), and was characterized by LE (seizures, cognitive disorders, behavioral changes), however, some rare manifestations, such as cerebellar ataxia and opsoclonus-myoclonus syndrome, were also described. In contrast to anti-LGI1 encephalitis, approximately 50% of anti-GABABR encephalitis patients have associated tumour, most commonly small cell lung cancer (SCLC). Anti-GABABR encephalitis patients present with a median age of onset 61 years (range: 16-77 years), and males and females appear to be equally affected (61).

1.1.4.4 Anti-AMPAR encephalitis

AMPAR is an ionotropic receptor of the glutamate receptors family, which mediates fast excitatory synaptic transmission in the brain, and also plays important role in synaptic plasticity, memory, and learning (62). Anti-AMPAR encephalitis, caused by autoantibody production against the GluA1 or GluA2 subunits of the AMPAR, was first described by Lai et al. in 2009 (63). The autoantibodies cause increased internalization and degradation of GluA1 and GluA2-containing AMPAR, leading to the selective decrease in the total surface amount and synaptic localization of the receptor (64). Affected patients present with a median age of onset 56 years (range: 23-81 years), and there is female predominance. Anti-AMPAR encephalitis usually manifests in the form of LE (65), however, other symptoms, such as acute psychosis, have also been reported (66). In approximately 56% of patients, the syndrome is paraneoplastic (2).

1.1.4.5 Anti-DPPX encephalitis

DPPX is a regulatory protein of the Kv4.2 potassium channels, which participate in somatodendritic signal integration and attenuation of back-propagation of action potentials (67). Anti-DPPX encephalitis was first described in 2013 by Boronat et al. (68) in a group of four patients presenting with protracted encephalitis characterized by CNS hyperexcitability, including hyperekplexia, myoclonus, tremor, or seizures, which was preceded by weight loss and diarrhea in 3/4 patients. Previously, it has been reported that anti-DPPX encephalitis patients present with a median age of onset 52 years (range: 13-76 years), and males appear to be more frequently affected (2). Hara et al. (67) investigated data of all previously reported 39 anti-DPPX encephalitis cases, and found that 67% of patients developed the following triad during the disease course: 1) weight loss / gastrointestinal symptoms, 2) cognitive-mental dysfunction, and 3) CNS hyperexcitability. In approximately < 10% of patients, the syndrome was associated with B-cell neoplasms (2, 69).

1.1.4.6 Anti-IgLON5 encephalitis

Anti-IgLON5 encephalitis was first reported by Sabater et al. in 2014 (70), characterized by autoantibodies directed against the extracellular epitopes of IgLON5, which is a neuronal cell adhesion molecule with unclear function. Nissen et al. (71) described anti-IgLON5 encephalitis in patients with a median age of onset 62 years (range: 45-79 years), and both sexes were found to be equally affected. The disease is characterized by the combination of sleep disorder

associated with one or more of the following symptoms: bulbar dysfunction, gait abnormalities, oculomotor problems, and cognitive decline can also be present in some cases (72). It has been described that only 11% of reported anti-IgLON5 encephalitis cases had a history of malignancy, with no clearly defined associated cancer type (71).

1.1.4.7 Anti-GABAAR encephalitis

The GABAAR is a ligand-gated chloride channel, which mediates fast inhibitory synaptic transmission in the CNS, and its pharmacologic or genetic alteration leads to seizure (73). Anti-GABAAR encephalitis has been first described by Petit-Pedrol et al. (74) in 2014, and since then autoantibodies have been reported against the α1, β3 and γ2 subunits of the receptor (74-76). Anti-GABAAR encephalitis patients present with a median age of onset 40 years (range: 2 months-88 years), and males and females appear to be equally affected (2). The syndrome is most commonly characterized by seizures (88%) and can be frequently accompanied by status epilepticus. Other symptoms, such as alteration of cognition (67%), behavior changes (46%), altered consciousness (42%), or abnormal movements (35%) could be also observed. In 77% of patients, brain MRI show multifocal unilateral or bilateral cortical and subcortical FLAIR signal abnormalities (2, 73). In approximately 27% of patients, the syndrome is associated with thymoma (2).

1.1.5 Treatment and recovery of neuronal cell surface-antibody mediated AE patients

The current treatment of AE includes immunotherapy and removal of the associated tumour (43, 65). Most AE patients are treated with first-line immunotherapy, including steroids, IVIG, or plasma exchange, and if there is no clinical response, second-line immunotherapy, rituximab or cyclophosphamide is applied. Recently, rituximab is increasingly used as initial treatment due to its efficacy in refractory cases and beneficial effect on the number of clinical relapses (43). It was reported that anti-LGI1 and anti-Caspr2 encephalitis might have a more indolent course, and anti-LGI1 encephalitis patients have a more rapid response to immunotherapy compared to other AE subtypes. However, regarding recovery, only 70% of anti-LGI1 encephalitis patients had substantial recovery at 24 months compared to 81% in anti-NMDAR encephalitis patients (43, 53). Anti-GABABR and anti-AMPAR encephalitis, which are frequently associated with tumour, have lower rate of response to immunotherapy (65).

However, prompt immunotherapy has been associated with a favorable outcome in all AE subtypes, with clinical relapses occurring in 12 to 35% of AE patients (2).

1.1.6. General overview of neuromyelitis optica spectrum disorder

Neuromyelitis optica (NMO) is a rare antibody-mediated inflammatory CNS disorder, which was recognized as a distinct disease entity and separated from multiple sclerosis (MS) over the past 10 years (77-81). The disease was traditionally considered to be monophasic, consisting of simultaneous bilateral optic neuritis and transverse myelitis, but in the 20th century relapsing cases were described (82). A major advance was the discovery of a unique biomarker, the pathological autoantibodies against the aquaporin-4 (AQP4), the most abundant water channel protein in the CNS, which are highly specific for clinically diagnosed NMO (83, 84). The term neuromyelitis optica spectrum disorder (NMOSD) was introduced in 2007, and included 1) AQP4-IgG-seropositive NMO patients with limited or inaugural forms of the disease, 2) typical NMO patients with cerebral, diencephalic, and brainstem lesions, 3) AQP4-IgG-seropositive patients with coexisting autoimmune disorders, and 4) patients diagnosed with opticospinal MS. The 2015 International Panel of NMO Diagnosis (IPND) criteria outlined a broader spectrum of NMOSD, subsumed the NMO term into the single descriptive term NMOSD, and in comparison with the 2006 Wingerchuk criteria, which exclusively included optic neuritis and transverse myelitis as clinical presentations of the disease, described six core clinical features (85).

In ≥80% of NMOSD, AQP4-IgG autoantibodies are present, and in approximately 10–40% of patients, who are AQP4-IgG seronegative, autoantibodies against the myelin oligodendrocyte glycoprotein (MOG) can be detected (86, 87). AQP4-IgG-positive NMOSD is primarily considered to be an autoimmune astrocytopathy, resulting in secondary oligodendrocyte and neuronal damage, whereas, MOG-IgG-seropositive NMOSD is characterized by primary demyelination (88).

NMOSD occurs worldwide and affects all ethnicities. Although, previous epidemiological studies reported of significant ethnical and regional differences in incidence and prevalence rates of NMOSD, suggesting that NMOSD is less frequent in Caucasian populations compared to Asian, African and Latin American populations. A study reported of 2.6-times higher prevalence of NMOSD in Martinique (in which 90% of the population is black) than in Olmsted County in the USA (in which 82% of the population is white) (89), indicating higher frequencies in non-white individuals. Recently, in Austria (0.71/100,000) and

Australia and New Zealand (0.7/100,000) large population-based studies using the 2015 IPND criteria reported lower prevalence in predominantly Caucasian populations (90, 91). However, recent Hungarian estimates in adult population represented higher prevalence (1.91/100,000) (92), which was still lower compared to estimates in Asian populations (Japan: 3.42/100,000; South Korea: 2.56/100,000) (93, 94). Studies investigating the latitudinal gradient in incidence and prevalence of NMOSD did not find similar evidence as observed in MS (91, 93). NMOSD can occur at any age (median age at onset: 40 years in AQP4-IgG seropositive patients, 31 years in MOG-IgG seropositive patients) (87, 95), and is more common in women, particularly the AQP4-IgG seropositive NMOSD (96, 97). Previous studies suggest the possible role of genetic and/or environmental factors, such as dietary factors, smoking, low vitamin D levels in the aetiopathogenesis of NMOSD (98-100). It has been described that acute attacks can be preceded by respiratory acute infections, however, no specific infections have been convincingly linked to disease induction and reoccurrence of attacks in NMOSD (87, 95).

In NMOSD, disability due to incomplete recovery from acute attacks, is measured using the Expanded Disability Status Scale (EDSS), which was originally developed for MS patients. However, the limitation of this scale in NMOSD is the lack of sufficient reflection on visual impairment.

1.2 B cell activation, natural autoantibodies and bacterial pathogens associated with autoimmune CNS disorders

1.2.1 Pathological role of B cells in NMOSD and MS

MS and NMOSD have been historically classified as T cell-mediated autoimmune diseases (101). However, the clinical success of selective B cell-targeting therapies, such as anti-CD20 antibodies in the treatment of MS and NMOSD highlighted the role of B cells in disease initiation and progression (102, 103). The success of anti-CD20 therapy is due to the extinction of B cells from blood and peripheral lymphoid organs (104). Studies focusing on how B cells contribute to the pathogenesis of MS and NMOSD, revealed that B cell properties such as antigen presentation and cytokine production, shape the immune response of other immune cells (T cells, myeloid cells) both in a pro-inflammatory and a regulatory manner. Besides these peripheral properties, antibody producing B cells play an important role within the CNS, which is suggested to be different between MS and NMOSD (105).

The following section discusses in details the contribution of B cells to the pathogenesis of MS and NMOSD in three aspects: 1) antigen-presenting B cells activate other immune cells, 2) pro-inflammatory and regulatory cytokine secretion of B cells, and 3) pathogenic role of antibody-producing plasma cells:

1) B cells are professional antigen-presenting cells (APC), which constitutively express major histocompatibility complex (MHC) class II and co-stimulatory molecules (CD40, CD80, CD86), which are required for efficient priming of naïve T cells. Several studies suggest that in MS, B cells activate CNS-infiltrating T cells, which drive inflammation in brain and spinal cord. Increased MHC class II, CD40 and CD80 molecule expression levels were detected in active MS B cells compared to healthy controls (HCs), suggesting their enhanced APC capacity (106, 107). Both peripheral and CNS B cells represented a shift toward antigen-experienced memory B cells, which indicates their chronic activation (108). Furthermore, functional studies showed that relapsing-remitting multiple sclerosis (RRMS) B cells induced proliferation and interferon-gamma (IFN-γ) secretion of potentially pathogenic $CD4^+$ T helper 1 cells ex vivo (109).

2) Besides the co-stimulatory signals, T cell activation also relies on cytokine milieu provided by the APC, such as interleukin (IL)-6 secretion of B cells, which promotes Th17 cell differentiation and inhibits regulatory T cell generation (110, 111). B cells are relevant source of pro-inflammatory and anti-inflammatory cytokines: activated B cells mostly secrete pro-inflammatory, whereas, naïve B cells, plasmablasts, and plasma cells produce anti-inflammatory (IL-10, IL-35, and transforming growth factor beta (TGF-β)) cytokines. Previous studies revealed abnormal cytokine profiles, and suggest inflammation-promoting role of B cells in MS. Isolated peripheral blood MS B cells secreted elevated amounts of pro-inflammatory cytokines (IL-6, lymphotoxin alpha and tumour necrosis factor alpha (TNF-α)) and decreased amounts of anti-inflammatory IL-10 cytokine compared to HCs (112, 113). Furthermore, MS patients showed increased frequency of pro-inflammatory cytokine (granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and TNF-α) expressing memory B cells, which therapeutic removal resulted in a diminished pro-inflammatory IL-6 response by macrophages in a GM-CSF-dependent manner (114). The regulatory role of anti-inflammatory cytokine secreting B cells might be impaired in MS as experimental autoimmune encephalomyelitis (EAE) and human studies suggest its role in limiting pathogenic T cell responses (115, 116).

3) The process of antigen presentation, not only activate T cells, but in turn induces B cell proliferation and differentiation into memory cells and antibody-producing plasma cells.

First evidence of pathogenic B cell activation in MS is the presence of OCB in the CSF (117). These intrathecal immunoglobulins are likely produced by plasma cells within the CNS (118). Despite the antibody-secreting plasma cells' ability to cross the blood-brain barrier (BBB) and interact with the peripheral immune system, these cells mainly accumulate within the CNS of MS patients, and their exact pathological role and the particular antigens antibodies recognize is still unclear. In contrast, autoantibodies against two different target antigens, AQP4 and MOG, were identified in NMOSD patients' sera. Interestingly, anti-AQP4 antibodies can be found in the serum, but antibody titers are relatively low or absent in the CSF, and OCB in CSF only occur in 15-30% of patients (119, 120). These facts suggest that in NMOSD B cells are first activated outside the CNS resulting in a humoral immune response against AQP4 in the periphery. However, there is new evidence in NMOSD that similar to MS, B cells are able to cross the BBB, leading to the presence of AQP4-specific B cells and plasma cells both in the blood and the CSF (121). Although, the circumstances under which AQP4-specific B cells and antibodies gain access to the CNS are not fully understood. Similar to AQP4-IgG antibodies, MOG-IgG antibodies can be found in the serum, but in the CSF, similar to OCB occur occasionally (86, 122, 123).

In summary, the most striking similarity between AQP4-IgG and MOG-IgG-seropositive patients, is the peripherally generated CNS-targeting antibody response, which is the main disease driver. The pathogenic role of the antibodies outside the CNS and the way these CNS antigens reach the periphery is uncertain, however, it is assumed that they are being drained from the CNS to the peripheral lymph nodes along the lymphatic vessels (124). In contrast, in MS, B cells have pathogenic effect both in the periphery and within the CNS, independent of CNS-specific peripheral antibodies. Activated B cells migrate through blood or lymph vessels into peripheral lymphoid organs, where they undergo further activation and maturation. CNS injury in MS is suggested to be driven by two independent, inflammatory processes: 1) de novo infiltration of immune cells from the periphery into the CNS, and 2) chronic progression caused by CNS-intrinsic inflammation, which is promoted by CNS-resident immune cells and CNS-trapped leukocytes (125). This shows a gradual shift of disease-driving B cell functions from the periphery to the CNS with disease progression, and suggests that B cells may be involved in cortical injury in MS. Due to the different contributions of B cells to the pathogenesis, NMOSD patients may require MS-independent therapeutic strategies (105).

1.2.2 Activation of natural antibody producing B cells via the TLR homologue CD180

Bacteria express pathogen-associated molecular patterns (PAMPs), which are recognized by the pattern-recognition receptors (PRRs) of cells of the innate immune system, leading to APC activation, which leads to directed immune responses against the pathogens via activation of autoreactive T and B cells (126). Toll-like receptors (TLRs) are PRRs of the innate immune system, which sense microbial structures and host-derived danger signals. Their role is the host defense against pathogenic microorganisms, and also participate in the restoration and maintenance of tissue homeostasis (127).

CD180, or RP105 (radioprotective 105 kDa), is a TLR-like molecule, lacking an intracellular Toll-IL-1R (TIR) signaling domain, and was originally identified as a B cell membrane protein mediating polyclonal B cell activation, proliferation and immunoglobulin production (127, 128). Later, CD180 was described as a TLR homologue, also expressed by monocytes and dendritic cells, and correlation between CD180 and TLR4 expression was also described. The accessory molecule of CD180, MD-1 is a homologue of the TLR4 accessory molecule, MD-2, which highlights the close relationship of CD180 and TLR4. CD180 and its accessory molecule, MD-1, inhibit directly the ability of the TLR4 signaling complex to bind microbial ligands, and also serves as a negative regulator of TLR4 responses of APC (127).

Previous studies have reported of the altered expression and functions of CD180 in a substantial proportion of circulating B cells in autoimmune diseases (127). CD180-negative B cells are seldom in healthy individuals (1.7±1.1%) (129). Interestingly, in B cell-related diseases the numbers of CD180-negative B cells is increased. In systemic lupus erythematosus (SLE) patients, approximately 16% of SLE B cells were CD180-negative, whereas, almost all HCs B cells expressed CD180 (129). Similar results have been observed in dermatomyositis and in Sjögren's syndrome patients (130, 131). Moreover, disease severity in SLE correlated with the amount of CD180-negative B cells in the peripheral blood (129, 132).

CD180-negative B cells are considered to produce autoantibodies and participate in the pathophysiology of human SLE. In the co-culture of SLE B cells and anti-CD3-activated T cells, CD180-negative B cells produced higher level of anti-double stranded DNA (dsDNA) compared to the CD180-positive B cells (133). Naturally occurring ligands of CD180 is unknown, CD180-mediated signaling in B cells is mainly derived from studies investigating the effect of CD180 crosslinking with monoclonal anti-CD180 antibody. Studies suggest that CD180 directly or indirectly by regulating TLR functions, augments TLR ligand-driven B cell activation. Cooperation of CD180 and TLR9 in the promotion of human B cell proliferation

has been described. $CD19^+$ peripheral blood B cells of HCs proliferated in response to anti-CD180 antibody and CpG (TLR9 ligand) treatment, and co-treatment synergistically enhanced $CD19^+$ B cell proliferation, which was observed in both naïve and memory B cells, the latter also exhibited synergistically enhanced IgM and IgG production (134, 135). A study using tonsillar B cells, reported that the natural autoantibody producing non-switched memory (NS) B cells showed the strongest activation after CD180 ligation, and their stimulation via CD180 resulted in enhanced natural autoantibody production. These findings suggest possible causality between lack of CD180 expression on B cells and dysregulated B cell functions in B cell-related disorders (136).

1.2.3 Natural IgM and IgG autoantibody production of B cells

Since the discovery of natural autoantibodies (nAbs) in the 1960s, great effort has been devoted to describing their origin, regulation, and function (137). It has been determined that nAbs are present in neonates (cord blood) and healthy adults in the absence of exogenous antigen stimulation or deliberate immunization, and that their reactivity profiles are remarkably conserved between individuals (138). NAbs serve as the first line of defense against infections. In healthy individuals, approximately 80% of all nAbs are IgM, but IgG and IgA classes are also present (139-141). NAbs are low affinity, polyreactive antibodies, which have the ability to recognize evolutionary conserved epitopes in foreign antigens.

Natural IgM antibodies are produced by the B1 lymphocytes and marginal zone B cells, while IgG antibodies are known to be produced via the T cell-dependent interactions of follicular B2 lymphocytes (142). Although, natural and pathological IgG antibodies may have similar characteristics, it is important to differentiate them. The presence of pathological IgG antibodies in the blood is usually considered to be the result of a pathological breakdown in self-tolerance, which is confirmed by the fact that many autoimmune diseases, such as SLE and Sjögren's syndrome are initiated or exacerbated by IgG autoantibodies to specific cellular and tissue components (143, 144). Similar to pathological IgG antibodies, titer of natural IgG autoantibodies fluctuates over time and is abundant in human sera, and influenced by age, gender and disease (142), suggesting their adaptive-like nature. The potential role of natural IgG antibodies in controlling inflammation and in the protection against pathogens was also described (137, 145). In contrast to natural IgG antibodies, it has been described that the self-reactive IgM autoantibody repertoire differentiates during the first years of life and remains relatively constant thereafter. Natural IgM antibodies have various functional roles in the

immune system, including regulation of B cell development, clearance of apoptotic debris as an anti-inflammatory effect, protection against autoimmune diseases, maintaining tissue homeostasis and immunological balance (146). Beneficial role of nAbs is supported by the therapeutic application of IVIG, which is rich in nAbs, in a large number of autoimmune and inflammatory diseases (147).

1.2.4 Bacterial pathogens as possible causative factors in NMOSD and MS

The list of pathogens associated to MS or NMOSD has grown significantly over the recent years. It is supposed that synergistic interactions between multiple pathogens may play role in the pathogenesis of MS and NMOSD. The suspected mechanism of action of pathogenic bacteria bridges innate and adaptive immunity. Regarding acquired immunity, it is hypothesized that bacterial structures can activate autoreactive T cells or produce autoantibodies via molecular mimicry. In innate immunity, TLRs combat the invading bacteria, and upon activation they release cytokines and chemokines, which mediate the adaptive immune responses (148). Increased TLR2 expression in MS demyelinating brain lesions suggest their involvement in the autoimmune CNS disorders (149). Several infectious pathogens, including viruses such as the Epstein–Barr virus (EBV), as well as bacteria like Chlamydia pneumoniae, Chlamydia trachomatis, Mycoplasma pneumonia, Helicobacter pylori, and Borrelia burgdorferi, have been identified, however, their roles in disease development remain controversial.

Chlamydiae are gram-negative obligate intracellular bacteria, which inhibit host cell apoptosis to escape from $CD4^+$ and $CD8^+$ immune recognition (150). It is supposed that Chlamydia pneumoniae and Chlamydia trachomatis can induce the production of cross reactive antibodies to myelin proteins (151). It was reported that Chlamydia pneumoniae can infect neuronal glia and neuronal ependymal cells in the CNS of mice (152), and also able to alter the permeability and cross the BBB (153), inducing persistent infection in the brain. Higher prevalence of Chlamydia pneumoniae intrathecal antibodies was reported in the CSF of MS patients than in HCs and were also detected in NMOSD, however, Chlamydia trachomatis antibodies were present in only 2% of MS patients (151, 154, 155). Although, Chlamydia pneumoniae association with MS is still controversial, and it was also suggested that it might not be a causative agent of MS, instead may act as a co-factor in disease development (156).

Mycoplasma pneumonia is a small prokaryotic organism, which adheres to host cells by specialized attachments and adhesin proteins, have cellular invasive capacity, and its

lipoproteins play a key role in infection and modulate immunity via TLR1 and TLR2 (157). Mycoplasma pneumonia IgG and IgM-seropositivity was found to be more frequent in female RRMS patients compared to HCs, suggesting the role of chronic mycoplasma infection in the development of MS among women (158). However, there was no evidence for the presence of bacteria in serum and CSF of MS patients upon targeting the Mycoplasma 16S rDNA gene (159, 160). A case report showed that the bacterium is capable of invading the CNS, which may result in demyelination (161).

Helicobacter pylori is generally considered a non-invasive bacterium, which colonizes the surface of gastric epithelial cells, however, in vitro studies showed that it can enter host epithelial and immune cells (162). Meta-analyses have shown negative correlation between the presence of bacteria and MS in western countries (163, 164), and higher frequency of acute Helicobacter pylori infection was reported in RRMS patients with stable phase (165). In Asian countries, higher prevalence of Helicobacter pylori antibodies were found in AQP4-IgG-seropositive NMOSD patients, but not in MS patients (155). A recent seroprevalence study detected antibodies against the vacuolating cytotoxin A antigen of Helicobacter pylori more frequently in secondary progressive multiple sclerosis (SPMS) patients compared to HCs, suggesting differences between MS subtypes (166). It is assumed that in case of persistent bacterial infection, bacterial antigens are able to stimulate the constant release of pro-inflammatory cytokines from immune cells, resulting in the loss of self-tolerance. Helicobacter pylori can exert these effects locally and directly via the CNS with modulation of the brain–gut axis (167).

Borrelia burgdorferi is the causative agent of Lyme disease, and is able to induce TLR2-dependent macrophage activation, and can also drive Th1-type T-cell immunity (168). Different data have been reported on the prevalence of Borrelia burgdorferi antibodies in MS: reports of 38.5% antibody positivity in MS patients were described (169), whereas, other studies detected lower antibody levels in MS (14.2%) compared to HCs (25.3%) (170). It is assumed that the presence of antibodies against Borrelia burgdorferi in MS patients does not prove that the bacterium is the cause of the disease, however, due to its MS resembling symptoms, it is important to be considered during the differential diagnosis (148).

2. FOCUS AND AIM OF THE STUDIES

2.1 Study 1: Single-center study of onconeural and neuronal cell surface autoantibody testing in Hungary

2.1.1 Theoretical background of the study

PNSs and AE are rare neurological disorders, data describing their prevalence and incidence is scarce. Autoantibody testing performed in sera (PNSs) and sera and/or CSF (AE) is crucial to differentiate between the two disorders. The Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary, was as an exclusive center for onconeural and neuronal cell surface autoantibody testing in Hungary from 2010 until 2018.

2.1.2 Hypothesis and aim of the study

The aim of the study was to retrospectively analyze the results of serum and CSF samples of patients with suspected PNSs and AE, obtained by the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary from 2010 until 2018. In the study, we aimed to determine national 1) prevalence, 2) incidence, and 3) age- and sex-based distribution of twelve types of onconeural autoantibodies (anti-Hu, anti-Yo, anti-Ri, anti-Ma2, anti-CV2/CRMP5, anti-amphiphysin, anti-Tr/DNER, anti-GAD65, anti-Zic4, anti-titin, anti-SOX1 and anti-recoverin), and six types of autoantibodies directed against neuronal cell surface receptors or their associated proteins (anti-NMDAR, anti-LGI1, anti-Caspr2, anti-GABABR, anti-AMPAR1, and anti-AMPAR2) in the Hungarian population. We further aimed to determine 4) whether neuronal cell surface autoantibody types were detected in serum and/or CSF.

2.2 Study 2: Clinical characteristics of neuronal cell surface antibody-mediated autoimmune encephalitis in a Hungarian cohort

2.2.1 Theoretical background of the study

AE can present with various neurological symptoms, caused by neuronal cell surface autoantibody production. Clinical symptoms, CSF findings, EEG and brain MRI abnormalities

can aid the diagnosis, which promotes early immunotherapy leading to favorable outcome (2). In our previous study, we found neuronal cell surface autoantibody positivity in 60 out of 1,034 patients with suspected AE in the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary from 2012 through 2018 as part of a Hungarian nationwide program. An online questionnaire in collaboration with neuroimmunologists from four major clinical centers of the region was used for data collection of neuronal cell surface autoantibody-mediated AE patients.

2.2.2 Hypothesis and aim of the study

The aim of the study was to retrospectively determine national 1) demographics, 2) prodromal symptoms, 3) clinical features, 4) tumour associations, 4) CSF findings, 5) EEG and 6) brain MRI results, 7) therapy and 8) prognosis of 35 patients diagnosed with neuronal cell surface autoantibody positivity (anti-NMDAR, anti-LGI1, anti-GABABR, anti-Caspr2) in the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary from 2012 until 2018. We further aimed to compare AE patients based on 9) the presence of signs of CNS inflammation, and 10) neuronal cell surface autoantibody types.

2.3 Study 3: Investigation of B cell abnormalities in patients with autoimmune CNS disorders

2.3.1 Theoretical background of the study

Studies focusing on B cell subpopulations and B cell activation in autoimmune CNS disorders are limited, and its precise role in disease development is still unclear. NMOSD is a rare, antibody-mediated CNS inflammatory disorder (88). Alterations of naïve and memory B cell subsets in NMOSD compared to MS and HCs have been studied with controversial results. Altered expression of the TLR homologue CD180 and its potential pathological role in B cell activation and autoantibody production have been described in autoimmune disorders (127). NS B cells are suggested to have potential role in natural autoantibody production, and CD180 ligation of NS B cells resulted in their activation and enhanced natural IgM autoantibody production (136). The potential causative role of Chlamydia pneumoniae, Chlamydia trachomatis, Mycoplasma pneumonia, Helicobacter pylori, and Borrelia burgdorferi have been

raised in NMOSD and MS (148). Connection between natural IgG autoantibodies and bacterial antibodies have been described in disorders, such as cardiovascular diseases and anti-measles IgG seropositive SLE patients (171, 172).

2.3.2 Hypothesis and aim of the study

The aim of the study was to determine 1) distribution, and 2) CD180 expression of peripheral blood naïve, double negative (DN), switched (S) and non-switched (NS) memory B cell subsets defined by CD19/CD27/IgD staining in NMOSD, and compare with MS and HCs. We further aimed to measure 3) anti-citrate synthase (CS) natural IgM/G autoantibody levels, and 4) infection-induced antibody levels, including anti-Chlamydia pneumoniae, anti-Chlamydia trachomatis, anti-Mycoplasma pneumonia, anti-Helicobacter pylori and anti-Borrelia burgdorferi antibodies in sera, which might be involved in the development of NMOSD or MS. We aimed to 5) assess the correlation between anti-CS natural IgG autoantibody levels and infection-induced antibody levels.

3. MATERIALS AND METHODS

3.1 Single-center study of onconeural and neuronal cell surface autoantibody testing in Hungary

3.1.1 Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the local ethical board, the Regional Research Ethics Committee of the Medical Center, University of Pécs (number of approval: RIKEB 6966/2017). Human serum and CSF samples were obtained with informed consent of all subjects involved in the study.

3.1.2 Samples tested for onconeural and neuronal cell surface autoantibodies

The retrospective statistical study was based on analysis of onconeural and neuronal cell surface autoantibody test results from serum and CSF samples of patients with suspected PNSs or AE. Samples were obtained by the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary, from various Hungarian neurological clinics from 2010 (onconeural autoantibody testing) and 2012 (neuronal cell surface autoantibody testing) until 2018 as part of a nationwide program. In the investigated period our laboratory has received 2543 serum samples from 2362 patients with suspected PNSs and 1247 sera and/or CSF samples from a total of 1034 patients with suspected AE. Autoantibody tests were ordered by neurologists based on clinical suspicion of PNSs or AE.

3.1.3 Line-immunoblot assays for onconeural autoantibody detection

In patients with suspected PNSs, two types of line-immunoblot assays with recombinant protein antigens were applied for onconeural autoantibody detection in sera. The first type of line-immunoblot assay was introduced in the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary in 2010, and the onconeural autoantibody panel included six types of antibodies: anti-Hu, anti-Yo, anti-Ri, anti-Ma2, anti-CV2/CRMP5 and anti-amphiphysin (EUROLINE Neuronal Antigens Profile 2, DL 1111 1601-2 G, Euroimmun, Lübeck, Germany). A second type of line-immunoblot assay was introduced in our laboratory in 2016, including twelve types of onconeural autoantibodies, with the

addition of anti-Tr/DNER, anti-GAD65, anti-Zic4, anti-titin, anti-SOX1 and anti-recoverin (EUROLINE Paraneoplastic Neurological Syndromes 12 Ag, DL 1111 1601-7 G, Euroimmun, Lübeck, Germany). Using these types of line-immunoblot assays, the advantage is that sera can be tested for six or twelve types of onconeural autoantibodies simultaneously.

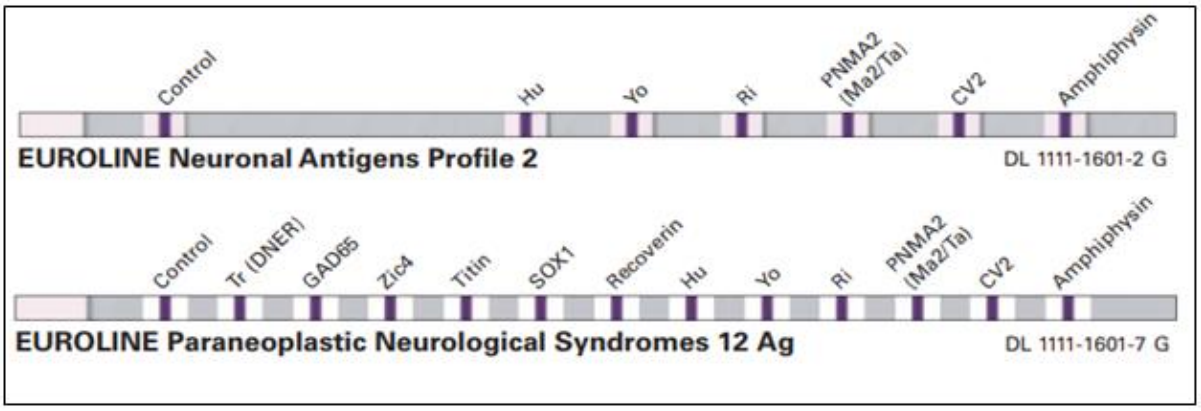


Figure 8. The two types of line-immunoblot assays with six (EUROLINE Neuronal Antigens Profile 2, DL 1111 1601-2 G, Euroimmun, Lübeck, Germany) and twelve (EUROLINE Paraneoplastic Neurological Syndromes 12 Ag, DL 1111 1601-7 G, Euroimmun, Lübeck, Germany) recombinant protein antigens used for onconeural antibody detection in sera of patients with suspected PNSs in the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary.

The immunoassays were optimized following the guideline included in the Manufacturer's Instruction. Blots were incubated with 1.5 ml Sample Buffer (included in the kit) for 5 min. After, 1.5 ml of sera (diluted 1:101) were incubated with the blots for 30 min at room temperature, followed by three washing steps with Wash Buffer (included in the kit) for 5 min. For secondary labeling, 1.5 ml of anti-human antibody (diluted 1:10; included in the kit) was applied for 30 min at room temperature, followed by the addition of 1.5 ml of substrate solution for 10 min at room temperature. After 3 washes, the line-blots were evaluated using the EUROLineScan detection system. Positive and negative controls were used to help evaluate the patient samples. Based on the intensity of the band staining, the EUROLineScan detection system assigned a value to each band using a range from 0 to 50: 0-5 negative, 6-10 equivocal, 11-25 mild positive (+), 26-50 (positive (++) and >50 strong positive (+++).

3.1.4 Indirect immunofluorescence BIOCHIP assays for neuronal cell surface autoantibody testing

In patients with suspected AE, three types of indirect immunofluorescence BIOCHIP assays were used for neuronal cell surface autoantibody detection in sera and/or CSF. The cell-based indirect immunofluorescence BIOCHIP assay was introduced in our laboratory in 2012 (Autoimmune Encephalitis Mosaic 1, FA 112d-1003-1, Euroimmun, Lübeck, Germany). On the BIOCHIP slide, HEK293 cells expressing six different antigens of interest (NMDAR, LGI1, Caspr2, GABABR, AMPAR1, and AMPAR2) are immobilized as a mosaic. Five samples can be investigated on a single slide; 1 mosaic is suitable for the detection of six types of autoantibodies. The advantage of this test is the capacity of detecting simultaneously the presence of six different types of autoantibodies in a single sample.

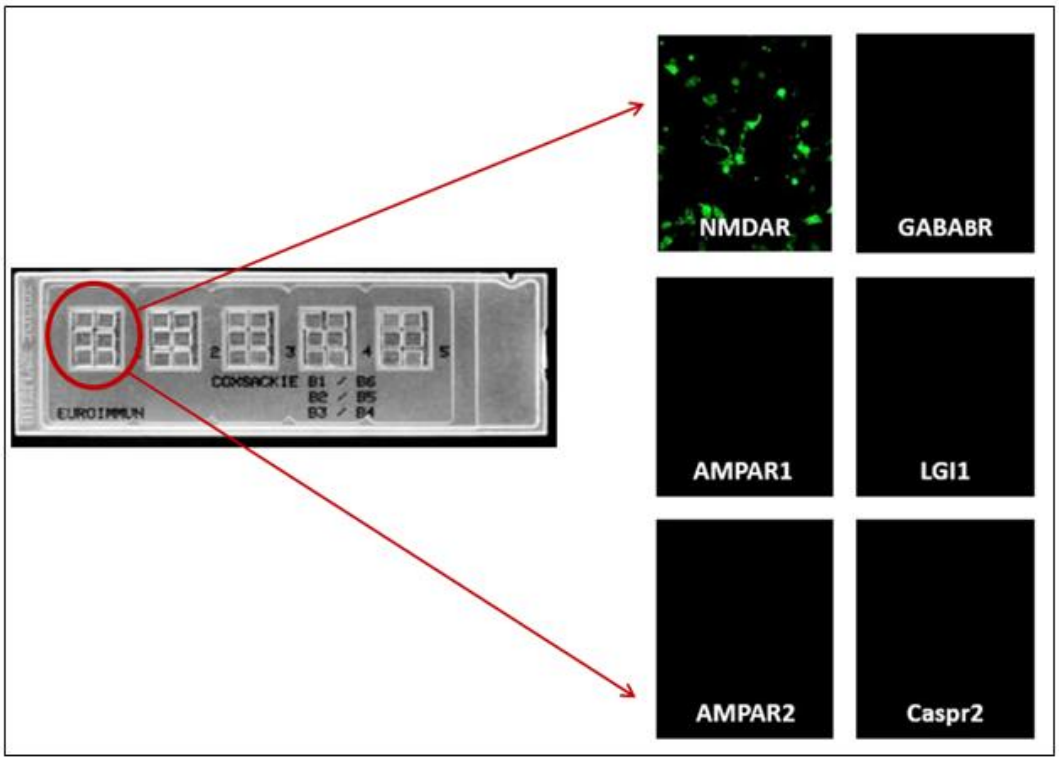


Figure 9. The cell-based indirect immunofluorescence BIOCHIP assay with HEK293 cells expressing six different antigens used for neuronal cell surface autoantibody detection in sera and/or CSF of patients with suspected AE in the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary.

In case of serum and/or CSF samples positive or equivocal for anti-NMDAR autoantibodies, a second type of indirect immunofluorescence BIOCHIP assay, containing fields of NMDAR expressing and control HEK293 cells was applied as a confirmatory test (The anti-Glutamate Receptor (type NMDA) IIFT kit, FC112d1005-51, Euroimmun, Lübeck, Germany). For histological localization of autoantibody positivity, a third type of indirect immunofluorescence BIOCHIP assay, containing rat brain tissue sections (hippocampus and cerebellum) was used for indirect immunofluorescence imaging (IIFT: Glutamate Receptor Mosaic 3, FA 111m-1003-3, Euroimmun, Lübeck, Germany).

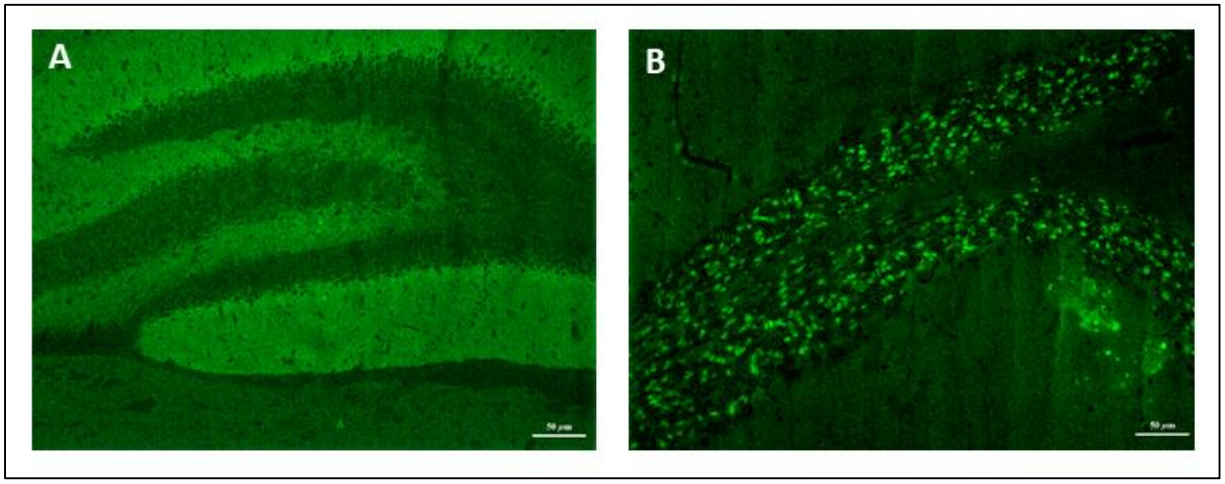


Figure 10. Positive indirect immunofluorescence staining of rat brain tissue section using indirect immunofluorescence BIOCHIP assay, CSF sample of an anti-NMDAR encephalitis patient was applied, and staining was performed in the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary: in the stratum moleculare layer of the hippocampus (neuropil-labelling; Panel A) and in the stratum granulosum layer of the cerebellum. Scale bars, 50 μm.

Optimization of the assays was based on the recommended protocol included in the Manufacturer's Instruction. 30 μl of the samples (sera diluted 1:10, or CSF undiluted) were incubated on the BIOCHIP containing the six transfected cell lines for 30 min at room temperature, followed by two washing steps with PBS-Tween 20 buffer (included in the kit) for 5 min. For secondary labeling, 25 μl of anti-human IgG (Fc specific)-FITC antibody specifically recognizing Fc fragment of all human IgG subclasses (IgG is the most frequently associated immunoglobulin isotype in AE (173), included in the kit), was applied for 30 min at

room temperature. After 2 washes for 5 min, glycerol (included in the kit) was used for covering the slides. Positive and negative controls were used to help evaluate the patient samples. Fluoresce imaging was performed using a fluorescence microscope (Olympus BX61) coupled with Zeiss Axiocam 305 color microscope digital camera and image processing system. The BIOCHIPs were evaluated independently by at least two laboratory specialists. Positive and negative controls were used, and reactions were graded as strong positive, positive, low positive, equivocal, and negative.

3.2 Clinical characteristics of neuronal cell surface autoantibody-mediated autoimmune encephalitis in a Hungarian cohort

3.2.1 Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the local ethical board, the Regional Research Ethics Committee of the Medical Center, University of Pécs (number of approval: RIKEB 6966/2017) and by the national ethical board, the Ethics Committee of the Medical Research Council of Hungary (number of approval: 49709-2/2019/EKU). Human serum and CSF samples were obtained with informed consent of all subjects involved in the study.

3.2.2 Patients

We retrospectively identified 35 patients with positive neuronal cell surface autoantibody (anti-NMDAR, anti-LGI1, anti-GABABR, anti-Caspr2) test results. Patients were selected based on our observational cohort study of patients tested positive for at least one neuronal cell surface autoantibody in sera, CSF or both in sera and CSF at the Department of Immunology and Biotechnology, Clinical Center, University of Pécs Medical School, Pécs, Hungary (**3.1.2 Samples tested for onconeural and neuronal cell surface and synaptic proteins autoantibodies** section)**.** Neuronal cell surface autoantibody detection was performed in the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary, as described earlier in **3.1.4 Indirect immunofluorescence BIOCHIP assays for neuronal cell surface autoantibody testing** section, and the antibody panel included anti-NMDAR, anti-GABABR, anti-AMPAR1, anti-AMPAR2, anti-LGI1 and anti-

Caspr2 autoantibodies. Of the total 35 patients, 24 were tested in CSF-serum pairs (68.6%), 9 for serum only (25.7%) and 2 for CSF only (5.7%).

Five patients with positive antibody results were excluded from further clinical analysis because of alternative diagnosis. Two patients with anti-Caspr2 positivity detected in sera but not in CSF, and one patient with anti-NMDAR positivity in CSF but not in serum had a final diagnosis of stroke (of which the latter patient died). One patient with anti-NMDAR positivity found in sera but not in CSF, and one patient with anti-Caspr2 positivity detected only in sera (CSF was not available) had a final diagnosis of multiple sclerosis.

Patients with positive neuronal cell surface autoantibody tests enrolled in this study fulfilled the criteria for definite AE defined by Graus et al (174): 1) subacute onset (rapid progression < 3 months) of working memory deficits (short-term memory loss), altered mental status, or psychiatric symptoms, and 2) new focal central nervous system (CNS) findings, seizures not explained by a previously known seizure disorder, CSF pleocytosis (white blood cell count > 5 cells/mm^3), or MRI features suggestive of encephalitis, and 3) reasonable exclusion of alternative causes. All anti-NMDAR autoantibody positive patients included in the study had ≥2 positive results during confirmatory tests (The anti-Glutamate Receptor (type NMDA) IIFT kit, FC112d1005-51, Euroimmun, Lübeck, Germany).

3.2.3 Data collection and analysis

Clinical data were collected using an online questionnaire in collaboration with neurologists specialized in neuroimmunology from four major clinical centers of the region in Hungary. Questions about demographics, prodromal symptoms, clinical features, CSF findings, EEG and brain MRI results, therapy and prognosis were included in the questionnaire.

We used the modified Rankin scale (mRS) to measure neurological outcome in AE patients (28, 175-177), which was originally developed to measure global disability after a stroke. The mRS scale is weighted toward motor function and evaluates the ability to walk to measure functional independence. In this study, the mRS score was determined at onset, at the time of the worst status of the patient, and at the last visit. The mRS score of 0-2 at the last visit was considered as good outcome and > 2 as poor outcome (177). Complete recovery was assessed in AE patients with mRS score of 0 at the last visit following treatment (median: 33 months; range: 1-77) (176). Relapse was defined as the new onset or worsening of symptoms, after at least 2 months of improvement or stabilization (178).

3.2.4 Statistical analysis

Statistical evaluation was performed with the SPSS IBM version 26 (IBM, Armonk, NY, USA). Categorical variables were described as percentages and numerical variables were described as medians and ranges. Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables were used as appropriate. *P*-values < 0.05 were considered statistically significant.

3.3 Investigation of B cell abnormalities in patients with autoimmune CNS disorders

3.3.1 Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the local ethical board, the Regional Research Ethics Committee of the Medical Center, University of Pécs (number of approval: RIKEB 7954/2019). Human serum samples were obtained with informed consent of all subjects involved in the study.

3.3.2 Patients

Twelve patients with neuromyelitis optica spectrum disorder (NMOSD), fifteen patients with RRMS (relapsing-remitting multiple sclerosis), and six age- and sex-matched healthy controls (HCs) were enrolled in the study. All NMOSD patients were diagnosed based on the 2015 new diagnostic criteria for NMOSDs (Table 5) and all MS patients met the revised McDonald criteria (Table 6). All 12 NMOSD patients included in our study were treated with immunosuppressive drugs, including steroid, azathioprine or tocilizumab and were considered to be treatment responders. From the total 15 RRMS patients included in our study, 14 patients were considered to be treatment responders. One RRMS patient was considered as treatment non-responder, and following peripheral blood sample taking, the patient's therapy was changed from fingolimod to natalizumab. Detailed patient data are summarized in Table 7.

Table 5. The 2015 International Panel for NMO Diagnosis (IPND) diagnostic criteria for NMOSD. *Data adapted from Wingerchuk et al. 2015 (85) and Jarius et al. 2020 (88).*

NMOSD with AQP4-IgG
Criteria A, and AQP4-IgG-positive serostatus
Criteria B, and **≥ 1 core clinical characteristics (may be the result of one or more clinical attacks) from below:** - acute optic neuritis - acute myelitis - acute area postrema syndrome - acute brainstem encephalitis - symptomatic narcolepsy or acute diencephalitis with MRI lesions: a) thalamus lesion, or b) hypothalamus lesion, or c) periependymal lesion at the third ventricle - acute (tel)encephalitis with MRI lesions: a) periependymal lesion at the lateral ventricles, or b) deep or subcortical white matter lesion, or c) longitudinally extensive (≥1/2 of its length) corpus callosum lesion, or d) longitudinally extensive (contiguously from the internal capsule to the cerebral peduncles) corticospinal tract lesion
Criteria C Exclusion of alternative diagnoses
NMOSD with negative or unknown AQP4-IgG serostatus
Criteria A, and Negative or unknown AQP4-IgG serostatus
Criteria B, and **≥ 2 core clinical characteristics (may be the result of one or more clinical attacks) from below, from which ≥ 1 has to be acute optic neuritis, myelitis or area postrema syndrome:** - acute optic neuritis with MRI lesions: a) normal or non-specific white matter lesions, or b) longitudinally extensive optic nerve lesion (≥1/2 of the distance from orbit to chiasm), or c) optic nerve lesion involving the optic chiasm - acute myelitis with MRI lesions: a) longitudinally extensive (≥ 3 vertebral segments) intramedullary spinal lesion, or b) spinal cord atrophy with or without T2 signal (≥ 3 vertebral segments) - acute area postrema syndrome with MRI lesions: a) dorsal medulla lesion, or b) area postrema lesion - acute brainstem encephalitis with MRI periependymal lesion at the fourth ventricle - symptomatic narcolepsy or acute diencephalitis with MRI lesions: a) thalamus lesion, or b) hypothalamus lesion, or c) periependymal lesion at the third ventricle - acute (tel)encephalitis with MRI lesions: a) extensive periependymal lesion at the lateral ventricles, or b) deep or subcortical white matter lesion, or c) longitudinally extensive (≥1/2 of its length) corpus callosum lesion, or d) longitudinally extensive (contiguously from the internal capsule to the cerebral peduncles) corticospinal tract lesion
Criteria C Exclusion of alternative diagnoses

Table 6. The 2017 revised McDonald diagnostic criteria for MS. *Data adapted from Thompson et al. 2018 (179) and Filippi et al. 2018 (180).*

Relapsing-remitting MS
≥ 2 clinical relapses and objective clinical evidence of ≥ 2 lesions with distinct anatomical location, **OR** ≥ 2 clinical relapses and objective clinical evidence of 1 lesion with historical evidence of a prior relapse involving a lesion in a distinct anatomic location
≥ 2 clinical relapses and objective clinical evidence of 1 lesion, **AND** **one criteria from below:** -DIS: second clinical relapse implicating a different CNS site -DIS: ≥1 symptomatic or asymptomatic T2-hyperintense lesions in ≥2 areas of the CNS: periventricular, juxtacortical/cortical, infratentorial or spinal cord
1 clinical relapse and objective clinical evidence of ≥ 2 lesions, **AND** **one criteria from below:** -DIT: second clinical relapse -DIT: simultaneous presence of both gadolinium-enhancing and non-enhancing symptomatic and asymptomatic lesions MRI lesions -DIT: new T2-hyperintense and/or gadolinium-enhancing MRI lesion compared to baseline scan (irrespective of the timing of the baseline MRI) -CSF: presence of oligoclonal bands
1 clinical relapse and objective clinical evidence of 1 lesion, **AND** **one criteria from below:** -DIS: second clinical relapse implicating a different CNS site -DIS: ≥1 symptomatic or asymptomatic T2-hyperintense lesions in ≥2 areas of the CNS: periventricular, juxtacortical/cortical, infratentorial or spinal cord **AND one criteria from below:** -DIT: second clinical relapse -DIT: simultaneous presence of both gadolinium-enhancing and non-enhancing symptomatic and asymptomatic lesions MRI lesions -DIT: new T2-hyperintense and/or gadolinium-enhancing MRI lesion compared to baseline scan (irrespective of the timing of the baseline MRI) -CSF: presence of oligoclonal bands
Primary progressive MS
Progression from onset, 1 year of disability progression (retrospectively or prospectively determined), **AND** **two criteria from below:** -≥1 symptomatic or asymptomatic T2-hyperintense lesions in ≥1 areas of the CNS: periventricular, juxtacortical/cortical, infratentorial -≥2 symptomatic or asymptomatic T2-hyperintense lesions in the spinal cord -CSF: presence of oligoclonal bands

Table 7. Clinical characteristics of neuromyelitis optica spectrum disorder (NMOSD), relapsing-remitting multiple sclerosis (RRMS) patients and healthy controls (HCs) involved in the study.

	HCs (n=6)	**NMOSD (n=12)**	**RRMS (n=15)**
Gender (female), n, %	4 (66.7%)	7 (58.3%)	13 (86.7%)
Anti-AQP4 antibody positivity, n, %	-	8 (66.7%)	-
Median age, y, range	47.5 (25-52)	50.5 (30-71)	42 (22-65)
Median age at onset, y, range	-	44 (16-69)	28 (12-42)
Median disease duration, y, range	-	8 (0.5-22)	15 (1-34)
No. of relapse, mean ± SD	-	2.5 ± 1.1	3.2 ± 1.8
EDSS, median, range	-	2 (0-6,5)	1.5 (0-8)
DMT drug *	-	-	10 (66.7%)
Immunosuppressive drug	-	12 (100%)	1 (6.7%) ♦

* DMT drugs in MS included dimethyl fumarate (4), interferon ß1a (2), fingolimod (2), glatiramer acetate (1) and alemtuzumab (1). *EDSS* = Expanded Disability Status Scale, *DMT* = disease modifying therapies.
♦ One RRMS patient received immunosuppressive therapy due to the first attack of the disease, first high dose parenteral steroid therapy was applied, followed by oral steroid treatment, which was ceased due to visual improvement of the patient.

3.3.3 Flow cytometric analysis of peripheral blood B cell subsets in NMOSD and MS

Flow cytometric analysis was performed to investigate distribution (NMOSD *n=12*, MS *n=15*, HCs *n=6*) and CD180 expression (NMOSD *n=9*, MS *n=7*, HCs *n=5*) of peripheral blood B cell subsets in NMOSD and MS patients. We used CD19 as a lineage marker of B cells (181), and CD27, which is considered as a universal memory B cell marker. Naïve B cells were characterized by the lack of CD27 expression (182). Upon analysis of distribution of memory B cell subsets, four B cell subpopulations were defined by CD27 and IgD labeling: $CD19^+CD27^+IgD^-$ switched memory (S), $CD19^+CD27^+IgD^+$ non-switched memory (NS), $CD19^+CD27^-IgD^+$ naïve and $CD19^+CD27^-IgD^-$ double negative (DN) B cells.

A four-color analysis was conducted using the combination of anti-human CD19-FITC (4G7, BD Biosciences Pharmingen, San Diego, CA, USA), anti-human CD27-APC (M-T271, BD Biosciences Pharmingen, San Diego, CA, USA), anti-human IgD-PerCP (IA6-2, BioLegend, San Diego, CA, USA) and anti-CD180-PE (G28-8, Becton Dickinson, Franklin Lakes, NJ, USA) antibodies, following the manufacturer's instructions. Peripheral blood samples were incubated with antibodies for 20 min. After hemolysis, cells were washed in phosphate-buffered saline (PBS), and fixed with FACSFix (0.5% PFA in PBS). Fluorescence

of labelled cells was recorded using BD FACSCalibur (BD Biosciences Pharmingen, San Diego, CA, USA), and analyzed with FCS Express 6 software (De Novo Software, Pasadena, CA, USA).

3.3.4 Naïve and memory B cell separation from PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Plaque Plus density gradient centrifugation of peripheral blood samples (NMOSD *n=5*, MS *n=5*, HCs *n=5*). PBMCs were washed twice in PBS and incubated with anti-human CD19-FITC (4G7, BD Biosciences Pharmingen, San Diego, CA, USA) and anti-human CD27-APC (M-T271, BD Biosciences Pharmingen, San Diego, CA, USA) antibodies for 30 min at 4°C, following the manufacturer's instructions. After the incubation period, samples were washed twice in PBS and taken up in an in-house buffer solution (containing PBS 1x, 0,5% BSA and 0,75% EDTA) and filtered through cell-strainer cap into Falcon polystyrene tubes under sterile conditions. Separation of naive ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells was performed using S3e Cell Sorter (Life Science Research/Bio-Rad Hercules, CA, USA). Bio-Rad Calibration Beads (ProLine Universal Calibration Beads, Life Science Research/Bio-Rad Hercules, CA, USA) were used to calibrate S3e Cell Sorter and gating was performed before the separation process in case of each samples. The purity of naïve and memory B cell subpopulations was checked using the BD FACSCalibur flow cytometer.

3.3.5 RNA isolation, cDNA synthesis, and qPCR from naïve and memory B cells

Total RNA was extracted from naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells immediately following their separation using the NucleoSpin RNA XS kit (Macherey-Nagel Inc, Bethlehem, PA, USA). Next, cDNA was generated with the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), and CD180 mRNA expression of naïve and memory B cells (NMOSD *n = 5*, MS *n =5*, HCs *n=5*) was determined by qPCR using the SensiFAST SYBR Lo-ROX Kit (Bioline, London, UK). Amplifications were performed using Applied Biosystems 7500 RT-PCR System (Thermo Fisher Scientific, Waltham, MA, USA), and CD180 gene expression was analyzed using 7500 Software v2.0.6 (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA expression of CD180 was normalized to GAPDH (a "housekeeping" gene) as reference, and fold changes (RQ) were calculated based on the 2-ddCT method.

3.3.6 ELISA measurements of natural IgM/G autoantibodies

An in-house ELISA method was applied for anti-citrate synthase (CS) IgM/G measurements. Nunc MaxiSorp™ ELISA plates were coated with Citrate Synthase from porcine heart (Sigma-Merck C3260) at a concentration of 2.25 µg/mL in Coating Buffer (Bio-Rad BUF030) (50 µL/well, 4-6°C, overnight). After blocking with 0.5 m/m% PVA (~72 000 Mw, 300 µL/well, room temperature, ≥2 hours), serum samples (NMOSD *n=10*, MS *n=13*, HCs *n=5*) were incubated (1:100 dilution) in washing buffer (100mM PBS, pH 7.4 + 1mL/L Tween 20) for 35 min at room temperature (standards, blanks, high and low controls were processed as patient sera). After 3 washing steps, anti-human IgM or IgG secondary antibody (Dako) was incubated for 30 min, followed by 3,3',5,5'-tetramethylbenzidine (TMB) substrate for 15 min and H_2SO_4 stop solution (50 µL/well) and reading was performed at λ = 450/620 using a Siemens BEP 2000 Advance® platform (Siemens AG, Frankfurt, Germany). Five-point dilution series of our in-house anti-CS standard was used for result quantitation, with subsequent 4-point sigmoid curve fitting.

3.3.7 ELISA measurements of bacterial autoantibodies

Commercial ELISA kits were used to detect bacterial autoantibodies in sera. Anti-Chlamydia pneumoniae IgM/G/A, anti-Chlamydia trachomatis IgM/G/A (NovaLisa, NovaTec GmBH, Dietzenbach, Germany), anti-Mycoplasma pneumonia IgM/G/A (VIROTECH Diagnostics GmbH, Rüsselsheim, Germany), anti-Helicobacter pylori IgG/A and anti-Borrelia burgdorferi IgM/G (Mikrogen GmBH, Neureid, Germany) autoantibodies were measured, according to the manufacturer's instructions. Serum samples (1:100 dilution) were incubated for 1 hour at room temperature, followed by the incubation of the plates with HRPO-conjugated anti-human IgA/IgG/IgM antibodies for 30 min at room temperature. Colour reaction was developed with TMB. Finally, stop solution was applied, and optical density was detected at 450 nm using a Siemens BEP 2000 Advance® platform (Siemens AG, Frankfurt, Germany).

3.3.8 Statistical analysis

Statistical evaluation was performed with the SPSS IBM version 26 statistics package (IBM, Armonk, NY, USA). Student’s t-tests, ANOVA, Mann-Whitney U and Kruskal–Wallis tests were used as appropriate, and p-values < 0.05 were considered statistically significant.

4. RESULTS

4.1 Single-center study of onconeural and neuronal cell surface autoantibody testing in Hungary

4.1.1 Prevalence, incidence and age- and sex-based distribution of onconeural autoantibodies

We obtained 2543 serum samples from 2362 patients with suspected PNSs from various neurology departments all over Hungary as a part of a nationwide program from 2010 until 2018. Of the 2543 samples, we found 235 positive samples (9.2%) belonging to 190 patients (8%). In our analysis, 151 serum samples were equivocal, belonging to 141 patients with suspected PNSs. Since the introduction of the onconeural autoantibody test in 2010, an overall increase was observed in the number of tested samples.

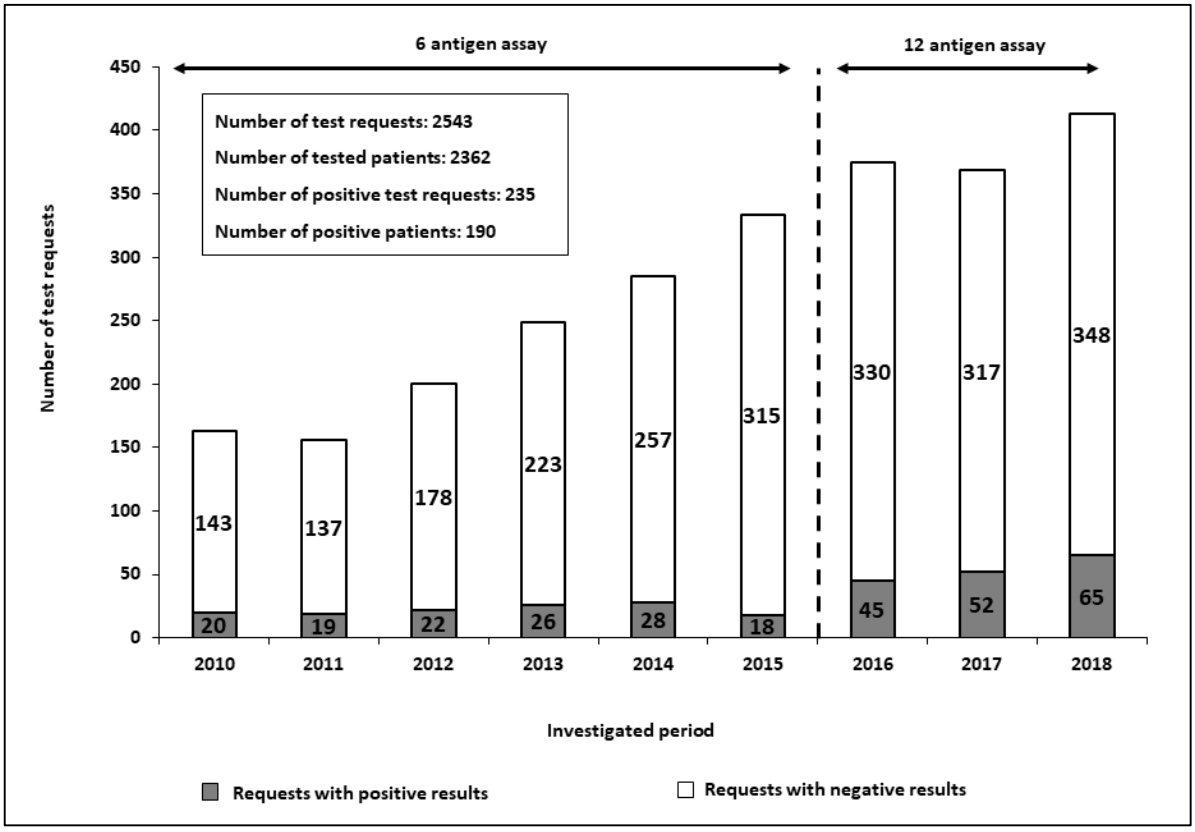


Figure 11. Annual distribution of onconeural autoantibody test requests from 2010 until 2018.

The frequency of positive samples varied over the eight-year investigated period: 12.3% (2010), 12.2% (2011), 11% (2012), 10.4% (2013), 9.8% (2014), 5.4% (2015), 12% (2016), 14.1% (2017), 15.7% (2018). The highest proportion of positive samples for onconeural antibodies was 15.7% (2018) and the lowest was 5.4% (2015) (Figure 11). From 2016, the number of tested samples and positive cases show larger increase compared to previous years. This increase might be the result of the introduction of the twelve-antigen line-immunoblot assay in 2016, making it possible to test a wider spectrum of onconeural autoantibodies.

We found an overall 1.9/100,000/year incidence of onconeural autoantibody positive cases in the Hungarian population. Further analysis of the onconeural autoantibody positive patient group (*n*=190) revealed the following autoantibody frequencies: anti-Yo (24.7%), anti-Hu (20%), anti-Ma2 (18.9%), anti-CV2 (12.1%), anti-titin (9.4%), anti-Zic4 (8.9%), anti-amphiphysin (7.4%), anti-Ri (2.6%), anti-GAD65 (2.6%), anti-Sox1 (2.6%) and anti-recoverin (1.1%). We haven't detected positivity against Tr/DNER. Twenty patients were positive for two types of onconeural autoantibodies simultaneously. Age- and sex-based distribution of the onconeural autoantibody positive patient group (*n*=190), revealed higher proportion of affected females (*n*=120) compared to males (*n*=70), with a median age of 62 years (range: 16-88 years). Data regarding incidence and age- and sex-based distribution for the twelve types of onconeural autoantibodies is summarized in Table 8.

Table 8. Incidence and age- and sex-based distribution of autoantibodies in the onconeural autoantibody positive patients (*n*=190).

Autoantibody	**Number of patients**	**Male/Female**	**Median age (range; years)**	**Incidence per 100,000 Person-Years**
Onconeural autoantibody positive patients from 2010 until 2018 (n_{tested}=2362 patients)				
Yo	47	0.5:1	63 (16-86)	0.47
Hu	38	0.7:1	64 (21-84)	0.38
Ma2	36	0.5:1	63 (19-88)	0.36
CV2	23	0.8:1	62 (23-73)	0.23
Amphiphysin	14	1:1	62 (35-74)	0.14
Ri	5	1:0.7	60 (49-60)	0.05
Onconeural autoantibody positive patients from 2016 until 2018 (n_{tested}=358 patients)				
Titin	18	0.6:1	66 (38-83)	0.18
Zic4	17	0.5:1	62 (32-79)	0.17
GAD65	5	0.7:1	61 (31-77)	0.05
Sox1	5	0.7:1	53 (46-61)	0.05
Recoverin	2	1:1	62 (58-66)	0.02
Tr/DNER	0	-	-	-

4.1.2 Prevalence, incidence and age- and sex-based distribution of neuronal cell surface autoantibodies

Since the introduction of neuronal cell surface autoantibody tests in 2012 in our institution until 2018, the number of test requests for diagnosing AE has increased each year. Our laboratory has received 1247 test requests (sera and/or CSF samples) from a total of 1034 patients for detection of AE-related autoantibodies. We found 98 positive samples belonging to 60 patients. This result reflects that autoantibodies were present in only 5.8% of the patients with clinically suspected AE. The frequency of the positive samples varied in the examination period: the highest ratio of positive test requests was during the first 4 years following the introduction of the test [22.4% (2012), 18.7% (2013), 6.1% (2014), 13.4% (2015)]. Although the number of test requests continued to increase, the ratio of positivity was lower during the past 3 years [4.3% (2016), 3% (2017), 4.4% (2018)]. The highest proportion of positive tests for AE was 22.4% (2012) and the lowest was 3% (2017) (Figure 12).

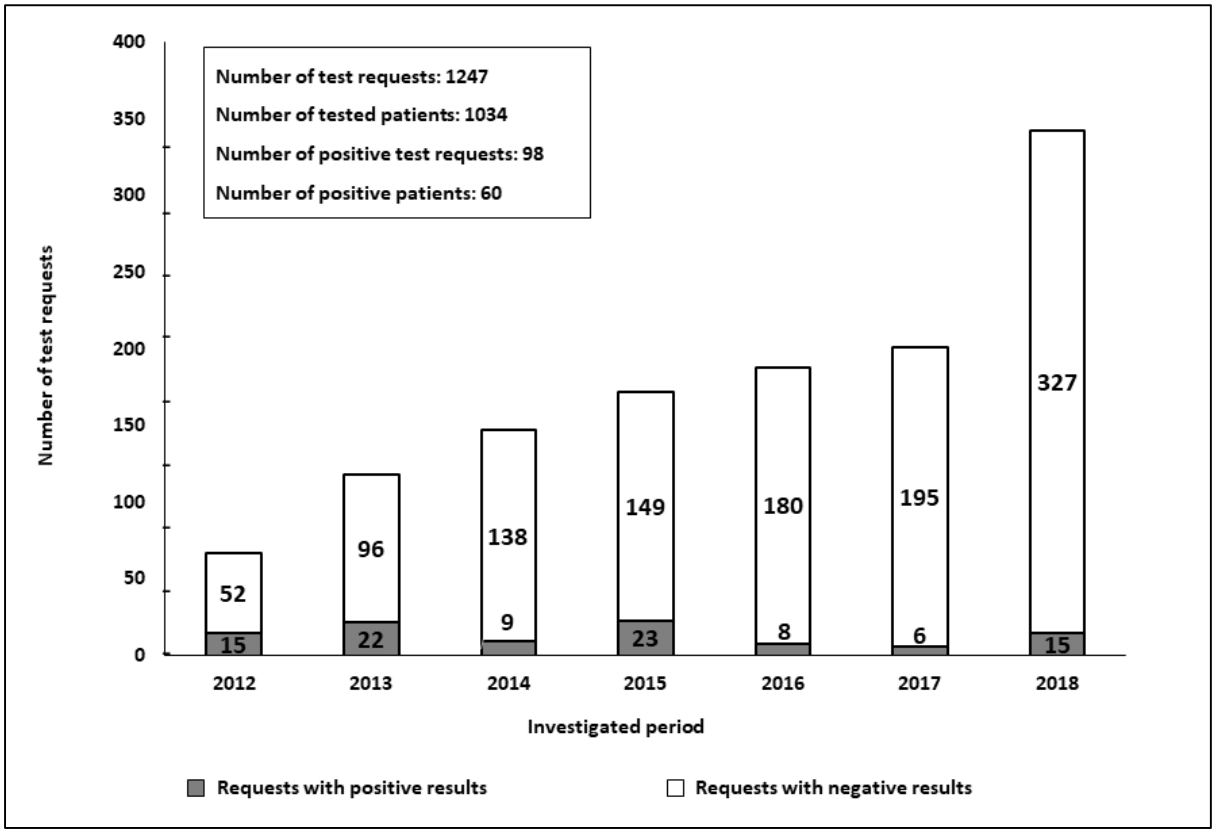


Figure 12. Annual distribution of neuronal cell surface autoantibody test requests from 2012 until 2018 (data of 12 equivocal test results are not shown).

We found an overall 0.6/100,000/year incidence of neuronal cell surface autoantibody positive cases in the Hungarian population. By analyzing the annual distribution of patients with positive AE test results, we found marked differences. The number of newly diagnosed patients was 3-14/year, while requests with positive results of the already diagnosed positive patients was only 1-4/year (data not shown). Figure 13 shows the annual distribution of positive patients with different types of autoantibodies. Patients with anti-NMDAR antibodies showed the highest frequency for each year during the examined period.

The frequency of different types of autoantibodies also varied: anti-NMDAR autoantibody was present in 70%, anti-LGI1 in 15%, anti-GABABR in 12%, and anti-Caspr2 in 7% of patients (Figure 14). Two patients showed positivity for two types of autoantibodies simultaneously (one patient showed positivity against LGI1 and Caspr2, and the other against GABABR and Caspr2). In 12 patients, the results obtained from sera were equivocal (11 anti-NMDAR, and 1 anti-GABABR); of which, 5 patients were negative upon simultaneous testing of CSF, and new samples from 3 patients were negative upon retesting.

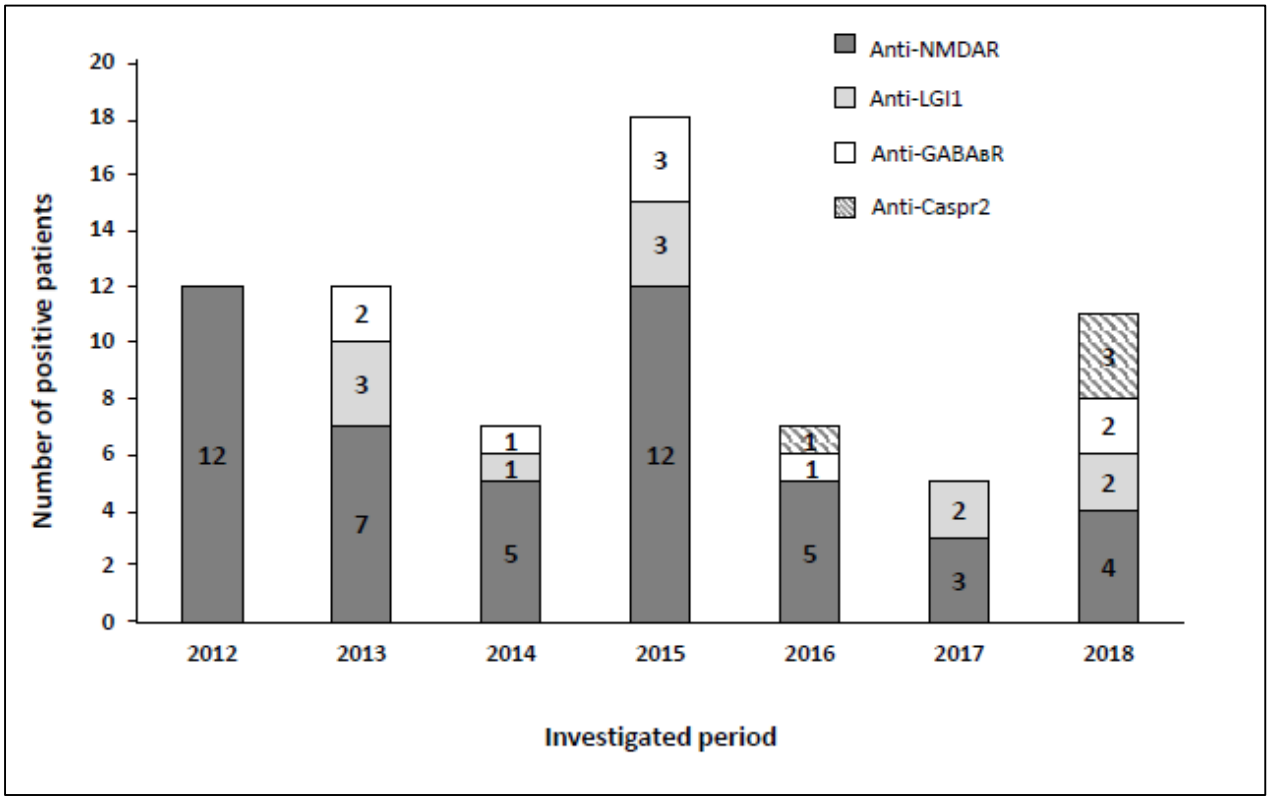


Figure 13. Annual distribution of autoimmune encephalitis-related autoantibody positive cases from 2012 until 2018.

Autoantibody	Number of patients (%)	Male/Female	Median age/range (years)	Incidence per 100,000 Person-Years
NMDAR	42 (70)	0.5:1	25 (1-75)	0.42
LGI1	9 (15)	1:0.6	51 (3-67)	0.09
GABAʙR	7 (12)	1:0.75	58 (16-81)	0.07
Caspr2	4 (7)	1:0	52 (3-72)	0.04
AMPAR1	0	–	–	–
AMPAR2	0	–	–	–

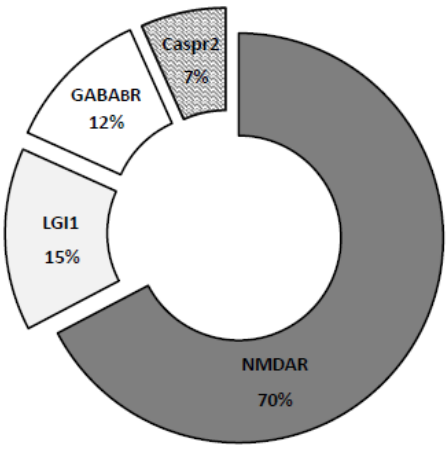


Figure14. Frequency and distribution of autoantibody types by age and sex in autoimmune encephalitis patients.

We investigated the distribution of AE-related autoantibody subtypes by age and sex (Figure 14). Anti-NMDAR encephalitis mostly affected females: of the 42 anti-NMDAR positive patients, 28 were female, median age 25 years. Anti-LGI1 encephalitis most frequently occurred in middle-aged males: of the 9 anti-LGI1 positive patients, 6 were male, median age 51 years. Anti-GABABR encephalitis affected elderly males: of the 7 anti-GABABR positive patients, 4 were male, median age 58 years. Anti-Caspr2 encephalitis occurred in male adults: of the 4 anti-Caspr2 positive patients, 3 were male, median age 52 years. Two patients showed positivity for two types of autoantibodies simultaneously: a 3-year-old boy was positive for both anti-LGI1 and anti-Caspr2, and a 60-year-old male was positive for anti-GABABR and anti-Caspr2.

Of the total 60 positive patients, in 30 cases repeated tests were performed at different time points (Table 9). In 17 cases, repeated laboratory tests resulted in autoantibody positivity multiple times, while in 13 patients the original positive autoantibody result subsequently switched to negative. Five patients (1 anti-NMDAR, 1 anti-LGI1, 1 anti-GABABR, and 1 positive for both anti-LGI1 and anti-Caspr2 antibodies) became negative within one year after the first positive test; 4 anti-NMDAR positive patients switched to negative within the 2nd year after the first positive test; 3 patients (2 anti-NMDAR and 1 anti-LGI1) turned to negative within the 3rd year; and one anti-NMDAR patient was found negative during the 5th year after the first positive test.

Table 9. Results of repeated test requests in 30 AE-related autoantibody positive patients.

Patients	ID	Autoantibody type	Follow-up time (years)	Number of test requests
Repeatedly positive	1.	NMDAR	1 (2013)	7
	2.		5 (2014-2018)	3
	3.		2 (2013-2014)	2
	4.		1 (2015)	2
	5.		2 (2015-2016)	2
	6.		1 (2012)	2
	7.		1 (2012)	2
	8.		4 (2012-2015)	2
	9.		1 (2018)	2
	10.		1 (2015)	2
	11.		2 (2016-2017)	2
	12.		1 (2015)	2
	13.		1 (2015)	2
	14.	LGI1	3 (2013-2015)	4
	15.	GABABR	3 (2013-2015)	3
	16.		1 (2015)	2
	17.		1 (2018)	2
Switched to negative	1.	NMDAR	5 (2012-2016)	7
	2.		3 (2016-2018)	4
	3.		2 (2012-2013)	2
	4.		1 (2017)	2
	5.		2 (2012-2013)	2
	6.		2 (2012-2013)	2
	7.		2 (2012-2013)	2
	8.		3 (2012-2014)	2
	9.	LGI1	1 (2013)	2
	10.		1 (2018)	2
	11.		3 (2013-2015)	3
	12.	LGI1+Caspr2	1 (2018)	3
	13.	GABABR	1 (2015)	2

4.1.3 Influence of sample type in neuronal cell surface autoantibody testing

In patients with suspected AE, generally antibody testing is preferred both from serum and CSF samples. Although, test characteristics show that some antibodies are more sensitive when testing serum (anti-LGI1, anti-Caspr2), whereas others are more sensitive when testing CSF (anti-NMDAR, anti-GABABR, anti-AMPAR). Based on this, we analyzed whether sera and/or CSF samples were more adequate for neuronal cell surface autoantibody testing.

Among the 60 positive patients, in 34 cases (57% of autoantibody-positive patients) autoantibodies were detected only in serum or only in CSF samples. In 28 cases (47%) only serum, while in 6 cases (10%) only CSF was tested. In 26 cases (43%) both sample types were investigated. Analyzing in more detail this group of autoantibody-positive patients, we found marked differences regarding the sample type and the strength of detected positivity in the different AE-related autoantibody types. Among the 17 anti-NMDAR patients – whose sera

and CSF were tested simultaneously – in 7 cases autoantibodies were detected in both serum and CSF, but in 3 cases stronger positivity was detected in the CSF. In 7 anti-NMDAR patients, positivity was found only in CSF, and in 3 cases only in serum. In 3 anti-LGI1 patients, we detected autoantibodies in sera only, but not in the CSF. In 4 anti-GABABR patients, positivity was detected in both serum and CSF, and in one case serum showed higher level of antibodies than CSF. In one anti-Caspr2 patient only the serum was positive. In one patient, both anti-LGI1 and anti-Caspr2 antibodies were detected in both sample types, although the anti-Caspr2 positivity was stronger in the serum than in CSF (Table 10).

Table 10. Summary of test results of autoimmune encephalitis-related autoantibodies in patients with serum and/or CSF positivity according to AE subtypes. *+++ strong positive, ++ positive, + low positive, +/- equivocal, - negative, NA: not available*

Patient ID	Serum	CSF	Autoantibody subtype
1.	+	NA	**NMDAR**
2.	+	+	
3.	+	NA	
4.	NA	+	
5.	+	NA	
6.	+	NA	
7.	+	NA	
8.	+	NA	
9.	+	NA	
10.	+	NA	
11.	+	+	
12.	+	NA	
13.	+	+	
14.	+	NA	
15.	+	NA	
16.	+	NA	
17.	+	+	
18.	+	+++	
19.	+	NA	
20.	-	+	
21.	NA	+	
22.	-	+	
23.	+	+++	
24.	-	+	
25.	+	NA	
26.	+/-	+	
27.	-	+	
28.	+	NA	
29.	-	+	
30.	NA	+	
31.	NA	+++	
32.	NA	+	
33.	+	++	
34.	+	NA	
35.	++	NA	
36.	NA	+	
37.	+	-	
38.	+	NA	
39.	+	-	
40.	-	++	
41.	+	-	
42.	++	NA	
43.	+	-	**LGI1**
44.	+	NA	
45.	+	NA	
46.	+	NA	
47.	++	NA	
48.	+	-	
49.	+++	NA	
50.	++	-	
51.	+	+	**LGI1 and Caspr2**
	++	+	
52.	+	+	**GABABR**
53.	+	+	
54.	+	+	
55.	+	NA	
56.	++	NA	
57.	++	+	
58.	+	NA	**GABABR and Caspr2**
	+	NA	
59.	+	NA	**Caspr2**
60.	+	-	

4.2 Clinical characteristics of neuronal cell surface autoantibody-mediated autoimmune encephalitis in a Hungarian cohort

4.2.1 Distribution of autoantibody positivity and demographic features

We identified and collected detailed clinical data of 30 patients with the diagnosis of definite AE. Of the 30 patients, autoantibodies were tested in CSF-serum pairs in 66.7%, only in serum in 26.7% and only in CSF in 6.7% of patients (Table 11). The most common antibody was anti-NMDAR (19/30, 63.3%), followed by anti-LGI1 (6/30, 20%), anti-GABABR (3/30, 10%) and anti-Caspr2 (3/30, 10%). One patient showed positivity for both anti-LGI1 and anti-Caspr2 antibodies. In the anti-NMDAR patient group, autoantibodies were detected in sera and CSF simultaneously in 31.6%, only in CSF in 36.8% and only in sera in 31.6% of patients. In the anti-LGI1 and in the anti-Caspr2 positive patients, in all cases autoantibodies were present only in sera, but not in the CSF with the exception of one patient with anti-LGI1 and anti-Caspr2 antibody positivity detected in both sera and CSF. In all anti-GABABR positive patients, we detected antibodies both in sera and CSF (Table 10). The group of 30 patients with definite AE, included 19 men (63.3%) and 11 women (36.7%) with a median age of 39.3 years (range: 1-75 years). Different sex ratios and median age were observed in the different AE types (Table 10).

Table 11. Demographic data and tested sample types in AE patients.

	AE (n=30)	**anti-NMDAR (n=19)**	**anti-LGI1 (n=6)**	**anti-GABABR (n=3)**	**anti-Caspr2 (n=3)**
Age (range; years)	39.3 (1-75)	32.5 (1-75)	46.8 (3-65)	47 (16-67)	47.7 (3-72)
Sex (male/female)	19:11	11:8	5:1	2:1	2:1
Complete recovery (n, %)*	20 (66.7%)	14 (73.7%)	4 (66.7%)	0	3 (100%)
Death (n, %)	3 (10%)	1 (5.3%)	1 (16.7%)	1 (33.3%)	0
Relapse (n, %)	1 (3.3%)	1 (5.3%)	0	0	0
Tested sample types					
CSF-serum pairs (n, %)	20 (66.7%)	12 (63.2%)	4 (66.7%)	3 (100%)	2 (66.7%)
Only CSF (n, %)	2 (6.7%)	2 (10.5%)	-	-	-
Only sera (n, %)	8 (26.7%)	5 (26.3%)	2 (33.3%)	-	1 (33.3%)

**Complete recovery was defined as the proportion of AE patients with mRS score of 0 at the last visit following treatment (median: 33 months; range: 1-77).*

4.2.2 Prodromal symptoms

Approximately 60% of AE patients present with prodromal symptoms, such as fever, headache, or malaise (2). In our cohort of Hungarian AE patients, fever or flu-like symptoms (10/30, 33.3%), occurring most frequently in anti-NMDAR encephalitis patients (8/19, 42.1%), were the most common prodromal symptoms. HSV encephalitis can trigger autoantibodies against the NMDAR, and approximately 20% of HSV encephalitis patients have relapsing neurological symptoms weeks after the onset of HSV encephalitis (2). In our cohort one anti-NMDAR patient had HSV encephalitis confirmed by positive PCR in the CSF, and anti-NMDAR encephalitis developed about a month after discharge from the hospital. Four additional patients with anti-NMDAR encephalitis had HSV IgM in the serum (Table 12).

Table 12. Prodromal symptoms and frequency of preceding HSV infection in AE patients.

	AE (n=30)	**anti-NMDAR (n=19)**	**anti-LGI1 (n=6)**	**anti-GABABR (n=3)**	**anti-Caspr2 (n=3)**
Fever/flu-like symptoms (n, %)	10 (33.3%)	8 (42.1%)	1 (16.7%)	1 (33.3%)	0
Headache (n, %)	3 (10%)	2 (10.5%)	0	1 (33.3%)	0
Vomiting (n, %)	1 (3.3%)	1 (5.3%)	0	0	0
HSV infection (n, %)	1 (3.3%)	1 (5.3%)	0	0	0
Systemic HSV IgM positivity (n, %)	4 (13.3%)	4 (21.5%)	0	0	0

4.2.3 Clinical symptoms and tumour associations

AE might present with a wide spectrum of clinical symptoms, including psychiatric symptoms, seizures (status epilepticus can also develop), cognitive impairment, speech disorders and insomnia. In the later phase of anti-NMDAR encephalitis, abnormal movements, such as orofacial dyskinesia, choreoathetosis and dystonia can also occur, and autonomic dysregulation might also be present. A significant proportion of anti-LGI1 encephalitis patients have hyponatremia and many patients develop FBDS prior to the development of LE, however, some rare manifestations, such as piloerection can also be noticed. Most AE patients present for psychiatric care, due to the development of prominent psychiatric symptoms within two weeks from disease onset. In our cohort, median time to diagnosis (onset of clinical symptoms until

positive autoantibody test result) was 2 months (range 1-53 months), and psychiatric symptoms were the most common initial presentations before the onset of neurologic dysfunction (17/30, 56.7%) (Table 13): 11 patients with anti-NMDAR, 3 patients with anti-LGI1, 2 patients with anti-GABABR and 2 patients with anti-Caspr2. During the disease course, psychiatric symptoms were altogether present in 25/30 (83.3%) of AE patients. In our cohort, psychiatric symptoms included the presence of one or more of the following: anxiety, apathy, aggressiveness, agitation, depression, hypersexuality, disorientation, visual/auditory hallucinations, behavioral changes, cognitive deficit, psychomotor retardation/agitation, catatonia, consciousness disorder, and mutism. The most common psychiatric symptom was disorientation (14/30), followed by visual/auditory hallucinations (7/30), psychomotor retardation (7/30) and behavioral changes (6/30) (Figure 15). In pediatric cases (age < 10 years: Patient 13, 14, 18 and 30), psychiatric symptoms included the presence of one or more of the following: disorientation, behavioral changes and catatonia.

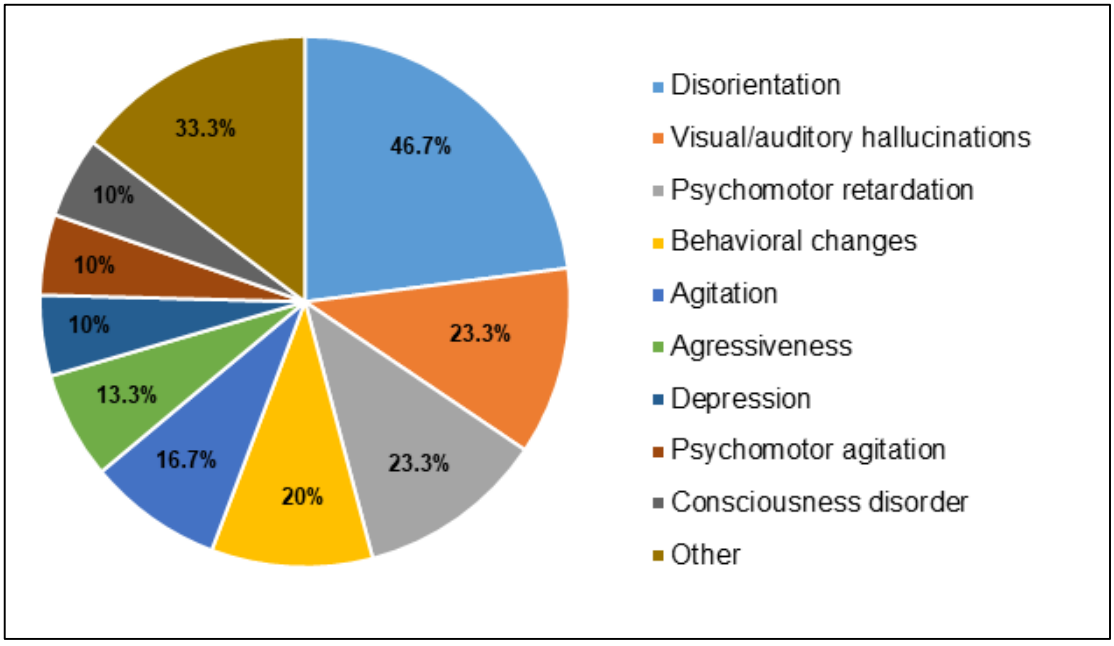


Figure 15. Psychiatric symptoms of AE patients. The diagram shows the distribution of psychiatric symptoms among the 30 AE patients in our cohort. Most common symptoms included disorientation, visual/auditory hallucinations, psychomotor retardation and behavioural changes. Frequency of rare psychiatric symptoms such as anxiety, cognitive deficit, catatonia, apathy, hypersexuality, and mutism are included in „Other".

Case number	Age	Sex (M/F)	Antibody	Clinical presentation*	CSF	MRI	EEG	Time to diagnosis (months)	mRS (diagnosis)	mRS (last visit)
1	24	M	NMDAR	Psychiatric symptoms, memory loss	OCB	BTH	Focal slowing	1	4	3
2	21	M	NMDAR	Psychiatric symptoms, insomnia, dyskinesias, seizures, memory loss	OCB+IgG index↑	BTH	Focal slowing	2	5	0
3	20	F	NMDAR	Psychiatric symptoms, seizures, memory loss	NL	NL	NL	4	3	0
♦ 4	23	M	NMDAR	Seizures, aphasia, memory loss	Pleo+TP↑	UTH	IEDs	2	2	0
5	32	M	NMDAR	Seizures	-	Unilateral hippocampal sclerosis	NL	2	2	0
6	70	F	NMDAR	Psychiatric symptoms, memory loss	NL	NL	NL	1	5	6
7	55	M	NMDAR	Memory loss, psychiatric symptoms	Pleo+TP↑	UTH	NL	3	3	2
8	75	F	NMDAR	Hyponatraemia, seizures, dyskinesias, psychiatric symptoms	TP↑	BTH	IEDs	1	3	0
9	48	M	NMDAR	Seizures, piloerection, hyponatraemia, psychiatric symptoms	OCB+TP↑	NL	NL	9	5	0
10	16	F	NMDAR	Psychiatric symptoms, seizures, dyskinesias, dysarthria	OCB	NL	NL	1	5	0
11	17	M	NMDAR	Seizures, status epilepticus, psychiatric symptoms, dyskinesias	NL	-	Diffuse slowing	1	5	1
12	17	F	NMDAR	Psychiatric symptoms, seizures, insomnia	NL	NL	NL	1	4	0
13	1	F	NMDAR	Psychiatric symptoms, seizures, ataxia, choreoathetosis, dyskinesias	NL	NL	NL	1	5	0
14	5	M	NMDAR	Ataxia, dysarthria, seizures, psychiatric symptoms, dyskineasias, dysautonomia	-	NL	Focal slowing	1	5	0
15	21	M	NMDAR	Psychiatric symptoms, memory loss, ataxia, cerebellar symptoms, neuropathy	-	Unilateral extratemporal hyperintensity	NL	-	5	0

16	29	F	NMDAR	Psychiatric symptoms, seizures, status epilepticus, dyskinesias, dystonia, memory loss	NL	NL	Diffuse slowing	-	5	0
17	70	M	NMDAR	Psychiatric symptoms, seizures	-	NL	Focal slowing	-	3	0
18	9	F	NMDAR	Psychiatric symptoms, seizures, status epilepticus, aphasia, dystonia	NL	NL	IEDs	-	5	0
19	65	M	NMDAR	Psychiatric symptoms, left central facial lesion, increased tone in extremities, right limb hypotonia	OCB	NL	IEDs	-	5	2
20	65	M	LGI1	Psychiatric symptoms, hyponatraemia, FBDS, seizures, memory loss	NL	UTH	NL	5	3	0
21	54	M	LGI1	Psychiatric symptoms, seizures, hyponatraemia, memory loss	NL	BTH	Ictal epileptiform discharges	53	3	2
22	51	M	LGI1	Memory loss, seizures, FBDS, hyponatraemia, insomnia, psychiatric symptoms	TP↑	NL	Ictal epileptiform discharges	36	3	0
23	58	M	LGI1	FBDS, hyponatraemia, dysautonomia, insomnia	TP↑	NL	NL	1	3	0
24	50	F	LGI1	Memory loss	NL	BTH	Focal slowing	1	3	6
25	67	M	GABABR	Psychiatric symptoms, memory loss	NL	UTH	NL	3	3	6
26	16	F	GABABR	Dyskinesias, memory loss, insomnia	TP↑	-	-	24	2	1
27	58	M	GABABR	Psychiatric symptoms, seizures, hyponatraemia	TP+IgG index↑	UTH	Focal slowing	7	5	4
28	72	M	Caspr2	Seizures, psychiatric symptoms, memory loss	TP↑	UTH	NL	1	3	0
29	68	F	Caspr2	Psychiatric symptoms, aphasia, seizures, status epilepticus	-	UTH	NL	9	4	0
30	3	M	LGI1+Caspr2	Psychiatric symptoms, seizures, hyponatraemia, insomnia, skin rashes	-	-	NL	1	4	0

Table 13. Clinical manifestations, auxiliary examinations and follow-up data of AE patients (*in order of onset of symptoms; ♦ HSV encephalitis preceded development of secunder anti-NMDAR encephalitis).

M: male; F: female; CSF: cerebrospinal fluid; NL: normal; OCB: oligoclonal band; Pleo: pleocytosis; TP: total protein; UTH: unilateral temporal hyperintensity; BTH: bilateral temporal hyperintensities; IEDs: interictal epileptiform discharge; mRS: modified Rankin scale score.

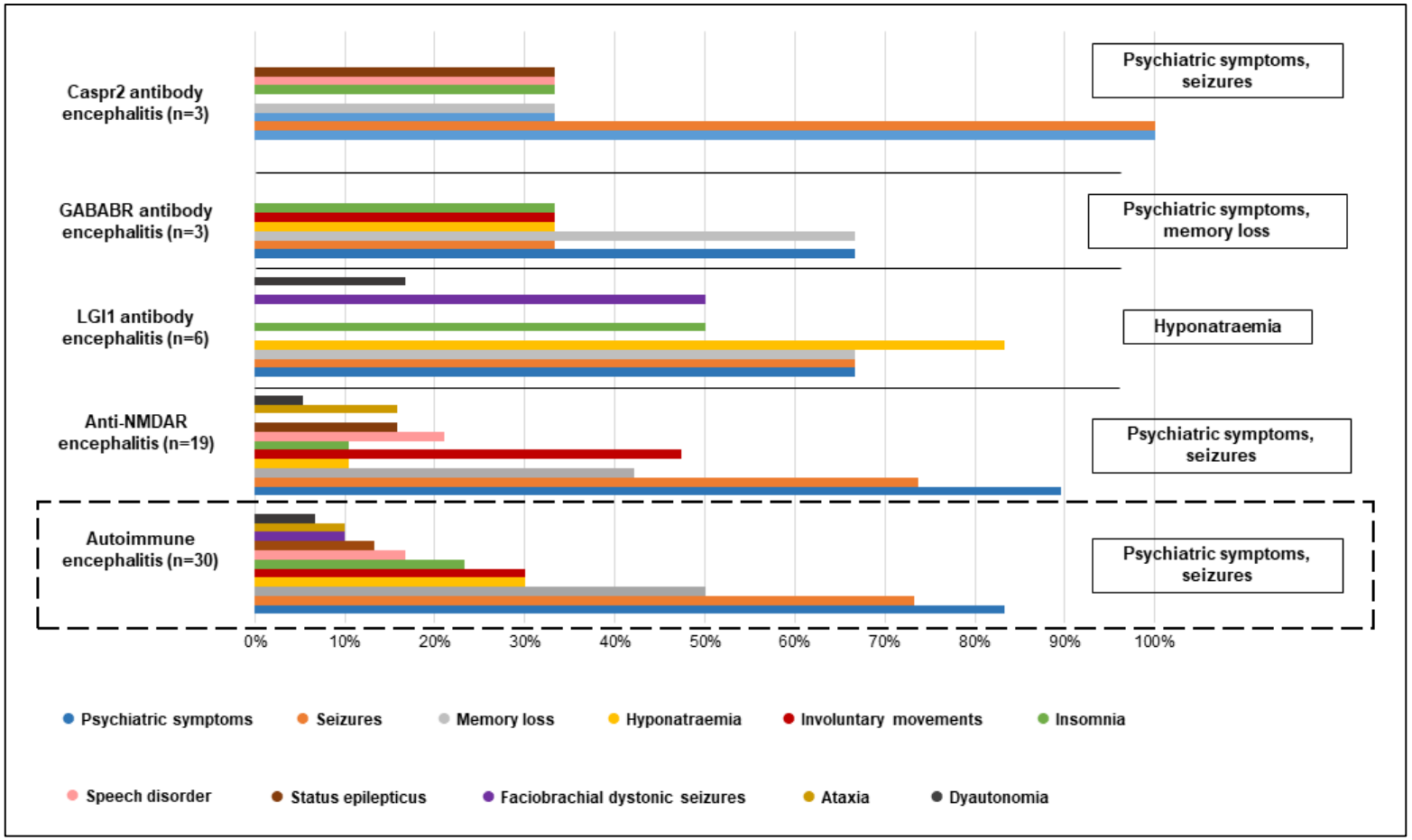


Figure 16. Characteristic clinical symptoms of AE patients. The bars show the frequency of different clinical features of AE patients. The diagram at the bottom shows that psychiatric symptoms and seizures were the most common in the 30 AE patients in our cohort. The other diagrams indicate distribution of clinical features separately in different AE types. Different distribution of clinical symptoms was observed in anti-LGI1 and anti-GABABR patients: in LGI1 encephalitis hyponatraemia and in GABABR encephalitis besides psychiatric symptoms memory loss were the most common symptoms.

Other clinical features included seizures (22/30), memory loss (15/30), insomnia (7/30), speech disorders such as dysarthria or aphasia (5/30), status epilepticus (4/30) and ataxia (3/30). Piloerection, cerebellar symptoms, neuropathy and skin rashes occurred in singular cases, respectively. Involuntary movements, such as dyskinesia, dystonia or choreoathetosis were the most common in anti-NMDAR encephalitis (9/19). Orofacial dyskinesia was present in 6/19 anti-NMDAR positive patients accompanied with hand dyskinesia in two patients. Dystonia occurred in two patients with anti-NMDAR encephalitis, and choreoathetosis was observed in one anti-NMDAR patient. Dysautonomia was present in one anti-NMDAR positive patient. Hyponatraemia (5/6) and FBDS (3/6) were the most frequent in the anti-LGI1 patient group. Clinical features of the different types of AE are summarized in Figure 16.

Table 14. Associated tumours in patients with AE. (* *compared to the time of positive neuronal autoantibody test result).* *LCNEC: large cell neuroendocrine carcinoma; DLBCL: diffuse large B-cell lymphoma.*

Antibody	Age (years)	Sex (M/F)	Tumour type	Tumour detection (months)*	Treatment	Outcome
NMDAR	70	F	LCNEC	87 (prior)	Tumour resection, chemotherapy, radiotherapy	Death
NMDAR	21	M	Chondroblastoma	33 (after)	Tumour resection	Complete remission
NMDAR	70	M	Prostate cancer	80 (after)	Radiotherapy	No remission
GABABR	76	M	SCLC	15 (after)	Chemotherapy	Death
GABABR	16	F	Thymoma	5 (after)	Treatment refusal	No remission
GABABR	58	M	SCLC	1 (after)	Chemotherapy	No remission
LGI1	50	F	Meningeal MALT-lymphoma	78 (prior)	Tumour resection, chemotherapy, radiotherapy	Complete remission
Caspr2	72	M	DLBCL	23 (prior)	Chemotherapy	No remission

Systemic tumours might have triggering role in the development of neuronal cell surface antibody-mediated AE, which is variably associated with different types of tumour depending on AE subtypes. Tumour association is rare in anti-LGI1 and anti-Caspr2 encephalitis, whereas, it is more common in anti-NMDAR and anti-GABABR encephalitis. Regarding associated tumours, eight cases (26.7%) were observed in our cohort: in 3 anti-NMDAR, 3 anti-GABABR, one anti-LGI1 and one anti-Caspr2 patients. Five patients were male (62.5%) and median age was 62.5 years (range: 16-72 years). The most common tumour type was lung carcinoma (LCNEC in one anti-NMDAR patient and SCLC in two anti-GABABR patient). In most cases (5/8, 62.5%), positive neuronal autoantibody test result preceded the detection of associated tumour. Median time from autoantibody positivity until detection of tumour was 15 months (Table 14).

4.2.4 CSF, EEG and brain MRI examinations

CSF examinations, including white blood cell count, presence of oligoclonal bands (OCB), protein level and IgG index, are important in the diagnosis of neuronal cell surface antibody-mediated AE, although, patients sometimes lack the abnormal CSF findings, especially anti-LGI1 and anti-Caspr2 patients (5). CSF was analyzed in 80% (24/30) of AE patients in our cohort (Figure 17). Abnormal CSF findings, such as pleocytosis (white blood cell count > 5 cells/mm^3), the presence of OCB, increased protein level (> 450 mg/L) and/or elevated IgG index (> 0.65) were observed in 13/24 of the tested AE patients. The majority of patients with anti-NMDAR (8/15) and anti-GABABR (2/3) had abnormal CSF results. Pleocytosis (2/15, 13.3%) and OCB (5/15, 33.3%) were detected exclusively in anti-NMDAR patients. Elevated IgG index was found in one anti-NMDAR and one anti-GABABR patients. Altogether, increased protein level was the most common abnormal CSF finding (9/24, 37.5%), accounting for all abnormal CSF cases in both the anti-LGI1 and anti-Caspr2 patient groups.

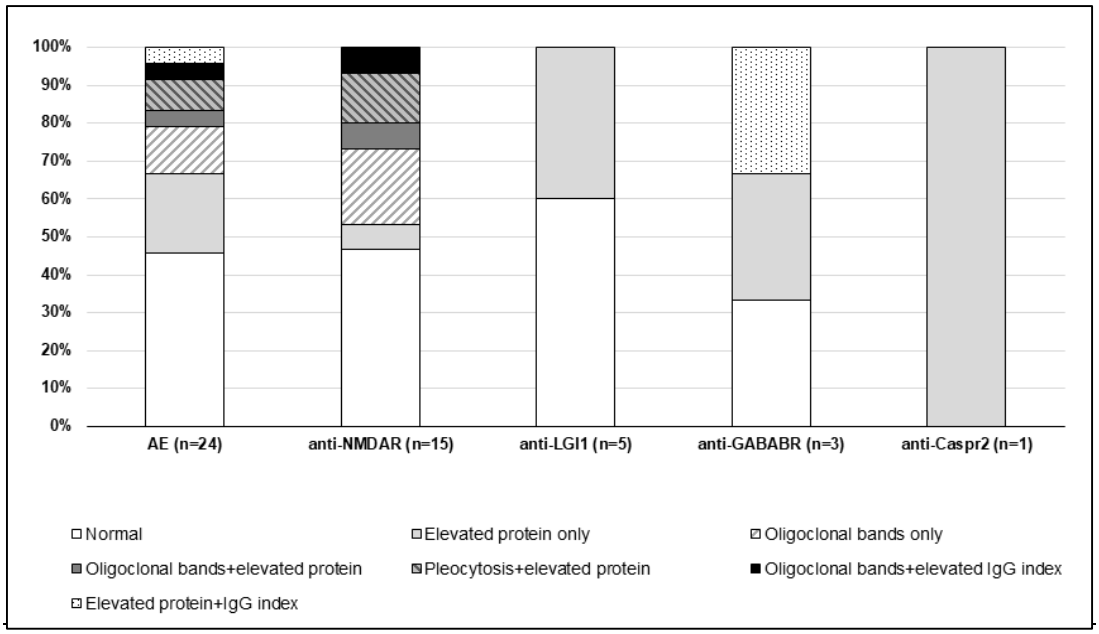


Figure 17. Composition of cerebrospinal fluid in AE patients. The bars demonstrate CSF analyses result of 24 AE patients from our cohort. At least one abnormal CSF finding was found in 13 AE patients, most commonly in anti-NMDAR and anti-GABABR patients. Pleocytosis and OCB were detected only in anti-NMDAR encephalitis.

The EEG is almost always (90–100%) abnormal in anti-NMDAR encephalitis patients, representing generalised or predominantly frontotemporal slowing, whereas, epileptogenic abnormalities are less common, occurring in 24–50% of patients, and are possibly more common during the early phase of the disease (183). We were able to evaluate EEG data of 29 AE patients. EEG abnormalities were observed in 14/29 patients (Table 15). The most common abnormal EEG findings were focal slowing (6/30) and interictal epileptiform discharges (4/30) (Figure 18).

Table 15. Detailed data of results of EEG evaluation in AE patients.

	AE (n=30)	**anti-NMDAR (n=19)**	**anti-LGI1 (n=6)**	**anti-GABABR (n=3)**	**anti-Caspr2 (n=3)**
Data not available	1	0	0	1 (33.3%)	0
Normal	15	9 (47.4%)	3 (50%)	1 (33.3%)	3 (100%)
Focal slowing	6	4 (21.1%)	1 (16.7%)	1 (33.3%)	0
Diffuse slowing	2	2 (10.5%)	0	0	0
Interictal epileptiform discharges	4	4 (21.1%)	0	0	0
Ictal epileptiform discharges	2	0	2 (33.3%)	0	0

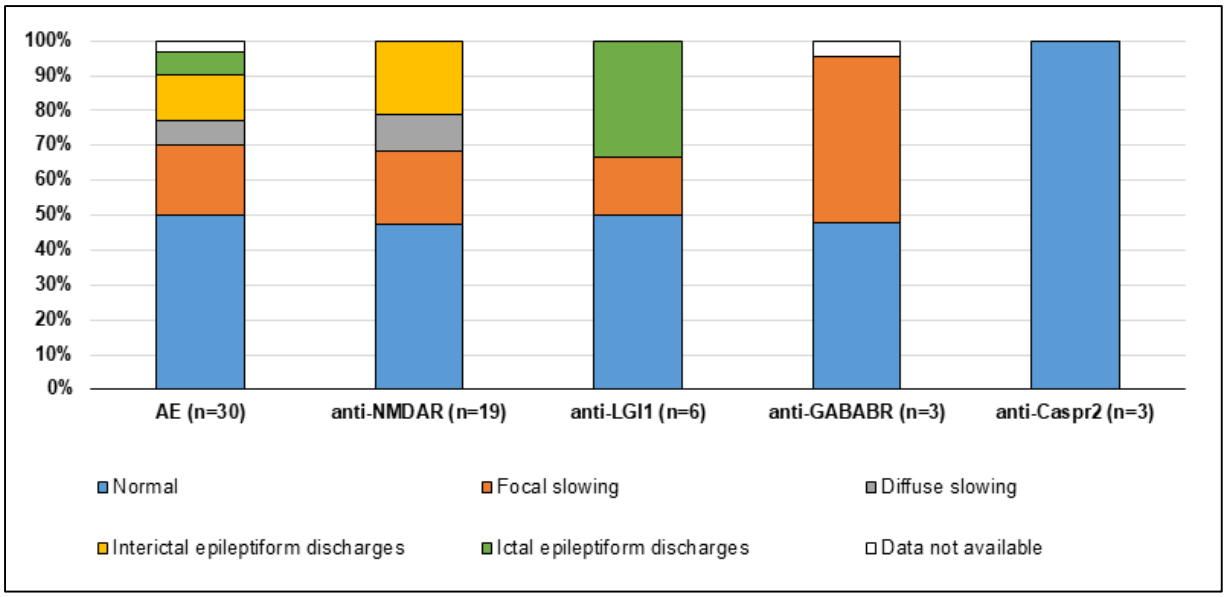


Figure 18. Characteristic EEG features of patients with different types of AE. The diagram shows EEG result of 29 AE patients from our cohort. In 14 AE patients, abnormalities were found, mainly focal slowing or interictal epileptiform discharges.

In approximately 60% of anti-NMDAR encephalitis patients, brain MRI is normal, and in other cases shows nonspecific findings, whereas, in anti-Caspr2 encephalitis patients it is considered to be frequently abnormal, but rarely suggestive of focal encephalitis (184). We evaluated brain MRI examinations in 27 AE patients. Abnormalities on brain MRI were observed in 14/27 patients (Table 16).

Table 16. Detailed data of results of brain MRI evaluation in AE patients.

	AE (n=30)	**anti-NMDAR (n=19)**	**anti-LGI1 (n=6)**	**anti-GABABR (n=3)**	**anti-Caspr2 (n=3)**
Data not available	3	1	1	1	1
Normal	13	11	2	0	0
Unilateral temporal hyperintensity (contrast enhancement)	7	2	1	2	2
Bilateral temporal hyperintensities (contrast enhancement)	5	3	2	0	0
Unilateral hippocampal sclerosis (contrast enhancement)	1	1	0	0	0
Unilateral extratemporal hyperintensity (no contrast enhancement)	1	1	0	0	0

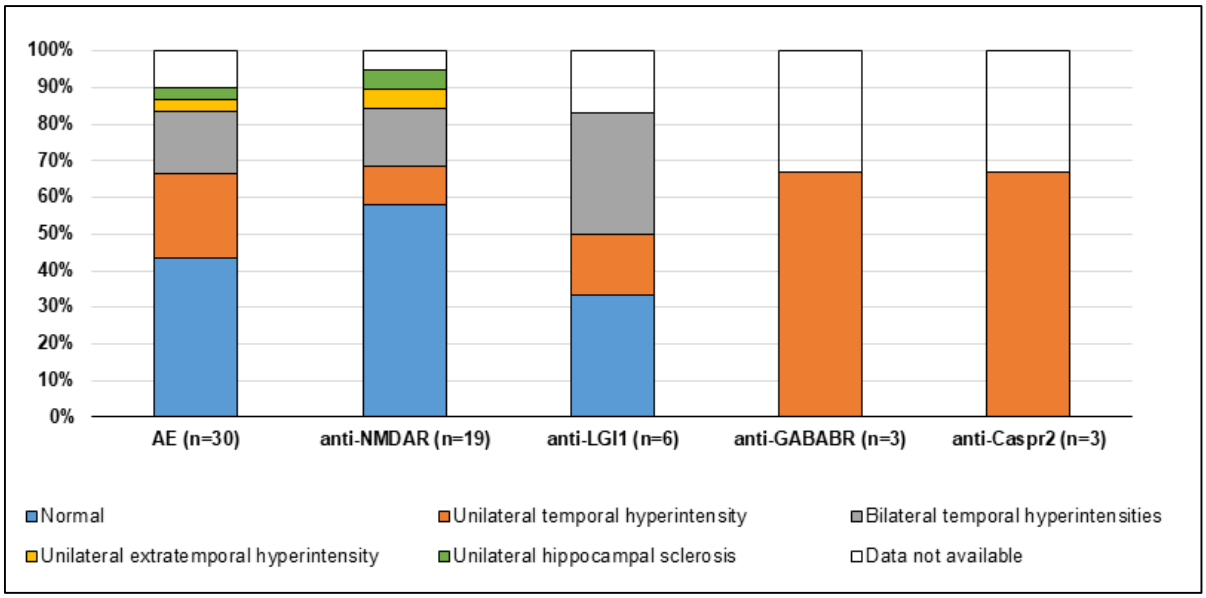


Figure 19. Brain MRI findings in AE patients. The diagram shows brain MRI result of 27 AE patients from our cohort. In 14 AE patients, abnormalities were found, mainly in the form of unilateral or bilateral temporal hyperintensities.

Abnormal brain MRI results were mainly unilateral or bilateral lesions in the insula/hippocampus (13/30) (Figure 19, Figure 20). In 7/14 (50%) of AE patients with abnormal brain MRI results, follow-up brain MRI examinations were conducted (median: 24 months, range: 2-37 months), which revealed hippocampal sclerosis or cerebral atrophy in three anti-LGI1 and two anti-NMDAR positive patients (5/7, 71.4% of patients with follow-up brain MRI result).

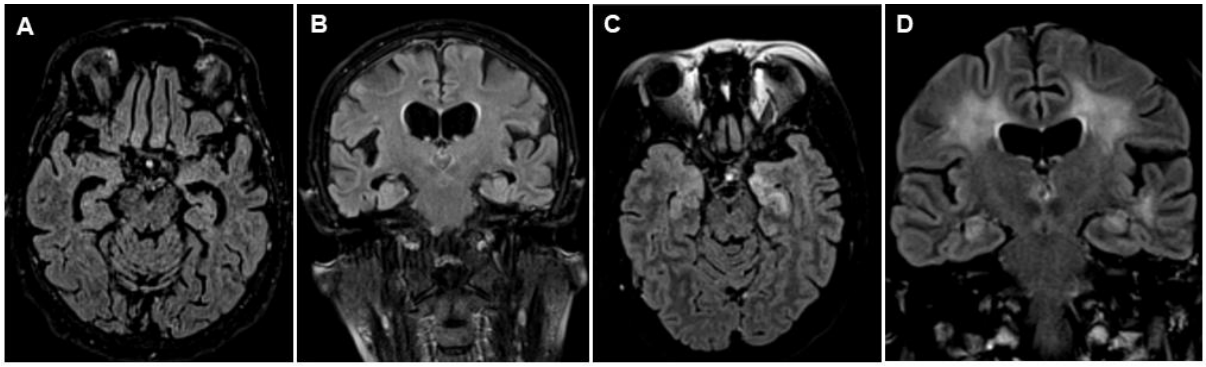


Figure 20. Axial and coronal FLAIR brain images from Patient 8 (Panel A, Panel B) and Patient 24 (Panel C, Panel D). Patient 8 was a 75-year-old female patient, who had anti-NMDAR autoantibody positivity detected in CSF and had hyperintensity and contrast enhancement within bilateral medial temporal lobes (Panel A-axial 3D FLAIR, Panel B-coronal T2 FLAIR). Patient 24 was a 50-year-old female patient, who had anti-LGI1 autoantibody positivity

detected in serum and had bilateral limbic encephalitis, dominant in the left temporal lobe, both on axial (Panel C) and coronal T2 FLAIR images (Panel D).

4.2.5 Treatment and outcomes

Besides removal of the systemic tumour, treatment of AE requires the administration of immunotherapy, which leads to favorable outcome in all AE subtypes. Anti-LGI1 and anti-Caspr2 encephalitis were described to have a more indolent course, however, higher rates of substantial recovery were noted in anti-NMDAR encephalitis patients compared to anti-LGI1 encephalitis patients.

In 24/30 of AE patients in our cohort, first-line immunotherapy was applied. Most commonly methylprednisolone pulse was used in combination with plasmapheresis (12/30). Other therapy included only the use of methylprednisolone (4/30) or in combination with intravenous immunoglobulin (5/30). Combination of all three types of immunotherapy was administered in 4/30 cases: three anti-NMDAR patients (mRS 5 at admission) presenting with psychiatric symptoms, seizures and involuntary movements, and one anti-LGI1 patient (mRS 3 at admission) with FBDS, hyponatraemia, dysautonomia and insomnia required such combination. All four AE patients had good outcome at the last visit (mRS 0). In one case, plasmapheresis was applied alone. Most patients (22/30, 73.3%) responded to the first-line therapy. Second-line therapy was introduced with azathioprine or rituximab or with the combination of the two in four cases with anti-NMDAR encephalitis presenting with severe disability during admission (mRS ≥ 4) and with no significant improvement to first-line therapy (Table 16). Azathioprin and/or rituximab was continued as maintenance therapy in three out of these four AE patients, due to residual symptoms (memory loss, psychiatric symptoms). Antiepileptics were used exclusively in 6/30 (20%) of AE patients, presenting predominantly with seizures and showing good outcome at the last visit (mRS median: 0; range: 0-1).

Introduction of immunotherapy was followed by at least one repeated neuronal cell surface autoantibody test in 20/30 (66.7%) of AE patients (median: 8.3 months, range: 1-47). Antibody positivity persisted in 11/20 (55%) of repeatedly tested patients (seven anti-NMDAR, three anti-GABABR and one anti-LGI1 encephalitis patients). The median hospital stay of AE patients was 23 days. Patients were severely impaired on admission, with a median mRS score of 4 (range 2-5). Most AE patients showed significant improvement after treatment and 25/30 (83.3%) achieved a good outcome (mRS ≤ 2). Median follow-up duration was 33 months (range

1-77) (Table 10). Five patients had a mRS ≥ 3 at the last visit, including one patient with anti-NMDAR and one patient with anti-GABABR who died of consequences of associated lung tumour and one patient with anti-LGI1 who died of deep vein thrombosis. One patient with anti-GABABR and small cell lung cancer was bedridden and required continuous nursing. One patient with anti-NMDAR had persistent cognitive deficit and memory loss, requiring some help as being unable to perform all previous activities. In our cohort, AE patients with associated tumour (*n=8*) had a significantly higher mRS score at the time of the last visit (median: 2.5, range: 6) compared to AE patients without tumour (*n=22*; mRS at the last visit median: 0, range: 3) (p = 0.045).

The rate of complete recovery in AE patients (follow-up median: 33 months; range: 1-77), showed favorable prognosis (20/30), with the highest rate in the anti-Caspr2 patient group. Relapses were uncommon in AE patients (1/30): only one male patient relapsed, whose anti-NMDAR positivity persisted in CSF during repeated autoantibody tests (follow-up: 47 months; number of autoantibody tests: 4). Death occurred in 3/30 of AE patients (Table 11).

Table 17. Immunotherapy of AE patients.

	AE (n=30)	**anti-NMDAR (n=19)**	**anti-LGI1 (n=6)**	**anti-GABABR (n=3)**	**anti-Caspr2 (n=3)**
First-line therapy	24 (80%)	14 (73.7%)	6 (100%)	2 (66.7%)	3 (100%)
Only steroid	4 (13.3%)	3 (15.8%)	1 (16.7%)	0	0
Only PE	1 (3.3%)	0	0	0	1 (33.3%)
Steroid+IVIG	3 (10%)	1 (5.3%)	1 (16.7%)	0	2 (66.7%)
Steroid+PE	12 (40%)	7 (36.8%)	3 (50%)	2 (66.7%)	0
Steroid+IVIG+PE	4 (13.3%)	3 (15.8%)	1 (16.7%)	0	0
Second-line therapy	4 (13.3%)	4 (21.1%)	0	0	0
Azathioprine	1 (3.3%)	1 (5.3%)	0	0	0
Rituximab	1 (3.3%)	1 (5.3%)	0	0	0
Azathioprine+Rituximab	2 (6.7%)	2 (10.5%)	0	0	0

4.2.6 Comparison of patients based on signs of CNS inflammation

Signs of inflammation in CSF and/or brain MRI may be absent in AE patients, especially in those over 60 years of age (185), which makes the diagnosis of AE complicated. Signs of CSF inflammation and brain MRI changes, particularly cerebellar atrophy in follow-up brain MRI negatively correlated with outcome of AE patients. Although, CSF and brain MRI abnormalities in the early phase of the disease did not show a strong relationship with patient

outcomes (177). We analyzed our cohort based on the presence of CNS inflammatory markers, including CSF inflammatory changes and/or brain MRI abnormalities. We defined CNS inflammation as the presence of at least one of the CSF inflammatory markers, such as pleocytosis (white blood cell count > 5 cells/mm^3), OCB, elevated protein or IgG index and/or brain MRI lesions suggestive of encephalitis (mesial temporal T2 signal hyperintensity or signs of demyelination). CNS inflammation was present in 19/30 patients (63.3%): both CSF and MRI abnormalities were detected in 7/30 patients (23.3%), 6/30 patients (20%) had brain MRI lesions suggestive of encephalitis without altered CSF findings, and 6/30 patients (20%) had signs of inflammation in CSF, but no abnormal brain MRI findings. In the remaining 11/30 cases, no changes indicating CNS inflammation were detected. Table 18 shows a comparison of clinical data of AE patients presenting with or without CNS inflammatory markers. In the patient group with inflammatory changes, age of onset was significantly higher ($p = 0.024$). No significant differences in sex, frequency of tumour association, time to diagnosis, prognosis and type of immunotherapy were observed between AE patients with and without CNS inflammatory markers.

4.2.7 Comparison of patients based on associated autoantibody types

It is well-known that besides the common clinical symptoms, treatment and outcome, AE subtypes are characterized by variable tumour associations, distinct auxiliary examination results, and in anti-LGI1 and anti-Caspr2 encephalitis a more indolent disease course have also been described compared to other AE subtypes. We also analyzed AE patients in our cohort based on the type of associated neuronal autoantibodies (Table 19). Patients were classified as anti-NMDAR positive, the most common AE subtype, and positive for other AE-related antibodies (anti-LGI1, anti-GABABR, anti-Caspr2). NMDAR encephalitis patients (*n=19*) were in more severe condition at the onset of the disease with significantly higher mRS score at the time of diagnosis (median: 5, range: 3) compared to LGI1, GABABR and Caspr2 encephalitis (*n=11*; mRS score at the diagnosis median: 3, range: 3) ($p = 0.028$). A trend of longer time to diagnosis was observed in the non-NMDAR patient group compared to NMDAR positive patients ($p = 0.063$). However, in two LGI1-encephalitis cases, final diagnosis of AE was retrospectively confirmed after the introduction of the cell-based assay in our laboratory in 2012. No significant differences were found in sex, frequency of tumour association, prognosis and type of immunotherapy between anti-NMDAR positive AE patients and patients positive for other AE-related antibodies (anti-LGI1, anti-GABABR, anti-Caspr2).

Table 18. Comparison of AE patients based on the presence of CNS inflammation.

Variables	**With CNS inflammation (n=19)**	**Without CNS inflammation (n=11)**
Age (range, years)	54 (16-75)	17 (1-70)
Male (n, %)	14 (73.7%)	5 (45.5%)
Tumour (n, %)	6 (31.6%)	2 (18.9%)
Diagnostic delay (median, months)	3	1
mRS at diagnosis (median)	3	5
mRS at last visit (median)	0	0
Status at Last Visit		
mRS 0-2 (n, %)	15 (78.9%)	10 (90.9%)
mRS 3-6 (n, %)	4 (21.1%)	1 (9.1%)
Immunotherapy		
Only steroid (n, %)	3 (15.8%)	1 (9.1%)
Steroid+IVIG (n, %)	0	2 (18.9%)
Steroid+PE (n, %)	10 (52.6%)	2 (18.9%)
Steroid+IVIG+PE (n, %)	1 (5.3%)	3 (27.3%)
Second-line therapy (n, %)	2 (10.5%)	2 (18.9%)

CNS inflammation was defined as the presence of at least one of the CSF inflammatory markers, such as pleocytosis (white blood cell count >5cells/mm3), OCB, elevated protein or IgG index and/or brain MRI lesions suggestive of encephalitis (mesial temporal T2 hypersignal or signs of demyelination). *CNS: central nervous system; IVIG: intravenous immunoglobulin; PE: plasmapheresis.*

Table 19. Comparison of AE patients based on the associated neuronal cell surface autoantibody type.

Variables	**anti-NMDAR encephalitis (n=19)**	**anti-LGI1, anti-GABABR, anti-Caspr2 encephalitis (n=11)**
Age (range, years)	32.5 (1-75)	58 (3-72)
Male (n, %)	11 (57.9%)	8 (72.7%)
Tumour (n, %)	3 (15.8%)	5 (45.5%)
Diagnostic delay (median, months)	1	5
mRS at diagnosis (median)	5	3
mRS at last visit (median)	0	0
Status at Last Visit		
mRS 0-2 (n, %)	17 (89.5%)	8 (72.7%)
mRS 3-6 (n, %)	2 (10.5%)	3 (27.3%)
Immunotherapy		
Only steroid (n, %)	3 (15.8%)	1 (9.1%)
Steroid+IVIG (n, %)	1 (5.3%)	1 (9.1%)
Steroid+PE (n, %)	5 26.3%)	5 (45.5%)
Steroid+IVIG+PE (n, %)	1 (5.3%)	1 (9.1%)
Second-line therapy (n, %)	4 (21.1%)	0

4.3 Investigation of B cell abnormalities in patients with autoimmune CNS disorders

4.3.1 Increased memory and decreased naïve B cell ratios in NMOSD compared to MS

Studies focusing on changes of B cell subpopulations in NMOSD and MS are limited and still remained controversial, and mostly focus on the alterations of B cell subsets caused by therapeutical effects. Alterations of B cell subsets have been described in NMOSD compared to MS and HCs. Increased double negative (DN) and decreased memory B cell ratios were reported in NMOSD compared to MS and HCs (121), and increased proportion of memory B cells was detected in AQP4-IgG-seropositive NMOSD, whereas, memory B cell ratios were unaltered in AQP4-IgG-seronegative NMOSD compared to HCs (80). In our study, upon flow cytometric analysis of B cell subsets, CD19 was used as a lineage marker of B cells (181), and CD27 was applied as a memory B cell marker. Naïve B cells were characterized by the lack of CD27 expression (182). For further analysis of memory B cell subsets, four B cell subpopulations were defined by CD27 and IgD labeling: $CD19^+CD27^+IgD^-$ switched memory (S), $CD19^+CD27^+IgD^+$ non-switched memory (NS), $CD19^+CD27^-IgD^+$ naïve and $CD19^+CD27^-IgD^-$ double negative (DN) B cells (Figure 21A).

First, we analyzed percentages of total CD19+ B cells in NMOSD and MS, which showed no significant differences compared to HC (NMOSD n = 12, median: 6.3, range: 1-20.7; MS n = 15, median: 7.3, range: 0.5-32.7; HC n = 6, median: 7.3, range: 3.8-12.7). Next, we compared the distribution of naïve and memory B cell subsets in NMOSD and MS. The ratio of naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells in NMOSD and MS showed no significant differences compared to HCs. However, in NMOSD frequency of naïve ($CD19^+CD27^-$) B cells was significantly lower, and percentage of memory ($CD19^+CD27^+$) B cells was significantly higher compared to MS (Figure 21B). Further, detailed analysis of the four B cell subpopulations revealed significantly lower percentage of naïve and higher frequency of NS, S memory and DN B cells in NMOSD compared to MS (Figure 21C). To investigate the potential effect of therapy on the distribution of B cell subsets, we compared proportion of B cell subpopulations between immunomodulatory treated (*n=11*) and untreated (*n=5*) MS patients, and found no significant differences (data not shown). Similar measurements were not applicable in NMOSD as all patients received immunosuppressive drugs.

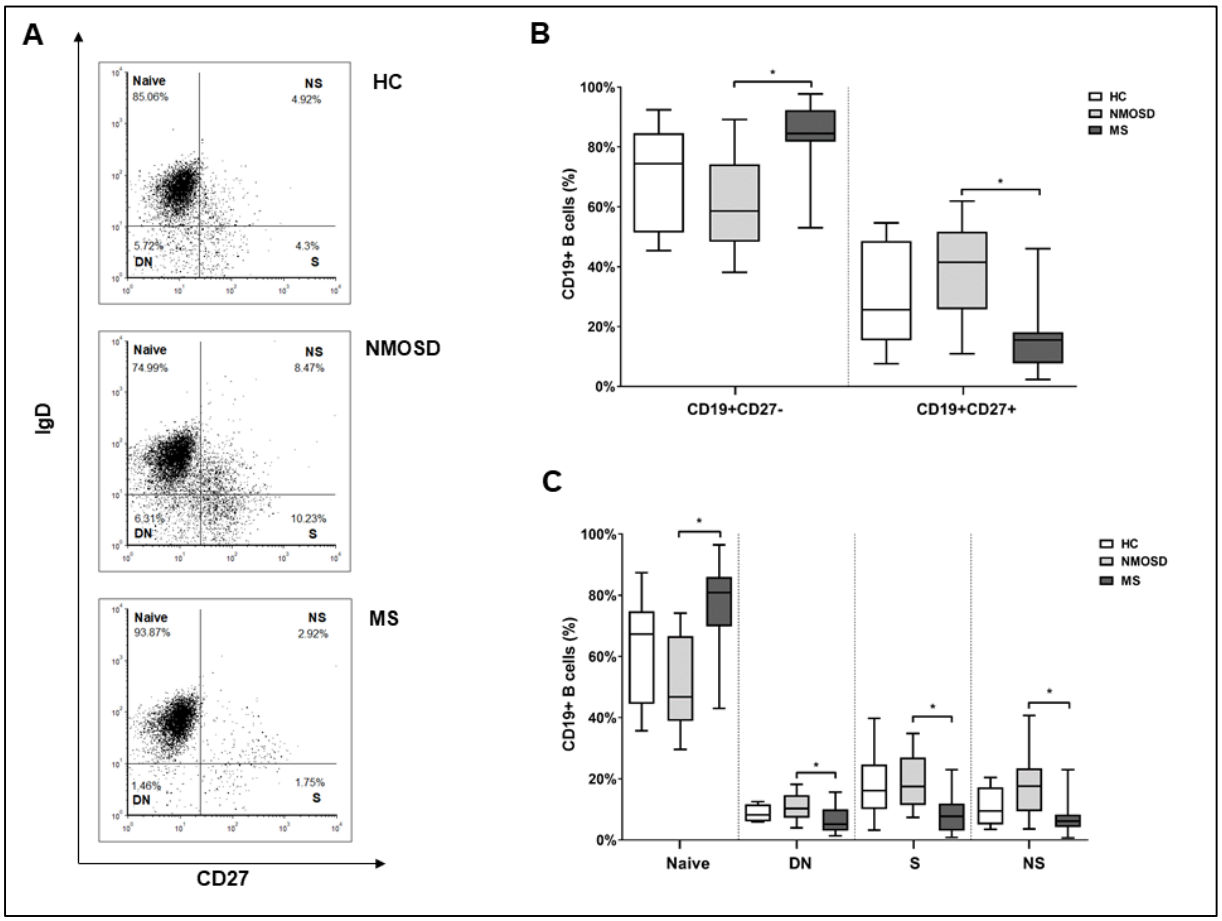


Figure 21. Analysis of B cell subsets in neuromyelitis optica spectrum disorder (NMOSD) and multiple sclerosis (MS) patients. Representative flow cytometry plots of four subsets of peripheral blood B cells defined by CD27 and IgD labeling: $CD19^{+}CD27^{-}IgD^{+}$ naïve, $CD19^{+}CD27^{+}IgD^{+}$ non-switched memory (NS), $CD19^{+}CD27^{+}IgD^{-}$ switched memory (S) and $CD19^{+}CD27^{-}IgD^{-}$ double negative (DN) B cells (Panel A). Flow cytometric analysis of naïve ($CD19^{+}CD27^{-}$) and memory ($CD19^{+}CD27^{+}$) B cells in peripheral blood of NMOSD, MS and healthy controls (HCs) (Panel B). Flow cytometric analysis of the defined four B cell subpopulations in peripheral blood of NMOSD, MS and HCs (Panel C). *Boxes show interquartile ranges (IQR); whiskers indicate lowest and highest values; horizontal lines represent medians, n = 6 HC, n = 12 NMOSD, n = 15 MS, * $p < 0.05$.*

4.3.2 Decreased CD180 expression of NS memory B cells in NMOSD and MS compared to HCs

The altered expression of CD180 in autoimmune diseases and its potential pathological role in B cell activation and autoantibody production have been already described (129, 131, 132, 186). Increased proportion of CD180-negative cells have been reported in autoimmune disorders, including SLE, Sjögren's syndrome and dermatomyositis. In our study, we measured CD180 expression at protein (mean fluorescence intensity, MFI) and mRNA (RQ) levels in separated naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cell subsets. We found no significant differences in any investigated B cell subsets among NMOSD, MS patients and HCs (Figure 22A, Figure 22B). We further analyzed the MFI of CD180 expression in $CD19^+CD27^-IgD^+$ naïve, $CD19^+CD27^+IgD^+$ non-switched memory (NS), $CD19^+CD27^+IgD^-$ switched memory (S) and $CD19^+CD27^-IgD^-$ double negative (DN) B cells, and found significantly decreased level of CD180 expression in NS B cells of both NMOSD and MS patients compared to HCs (Figure 22C).

4.3.3 Diminished IgM natural autoantibody level in NMOSD and MS compared to HCs

Previously, it has been reported that B cell stimulation via CD180 resulted in strong activation of NS B cells, along with a significant decrease in their CD180 expression and induction of natural autoantibody production (136). Altered natural autoantibody (anti-citrate synthase (CS)) levels in patients with different systemic autoimmune diseases have been reported (172). Consequently, we measured anti-CS IgM/G natural autoantibody levels in sera of NMOSD and MS patients and HCs. Anti-CS IgM level was significantly decreased in NMOSD and MS samples compared to HCs (Figure 23A), but no differences were found in anti-CS IgG levels (Figure 23B).

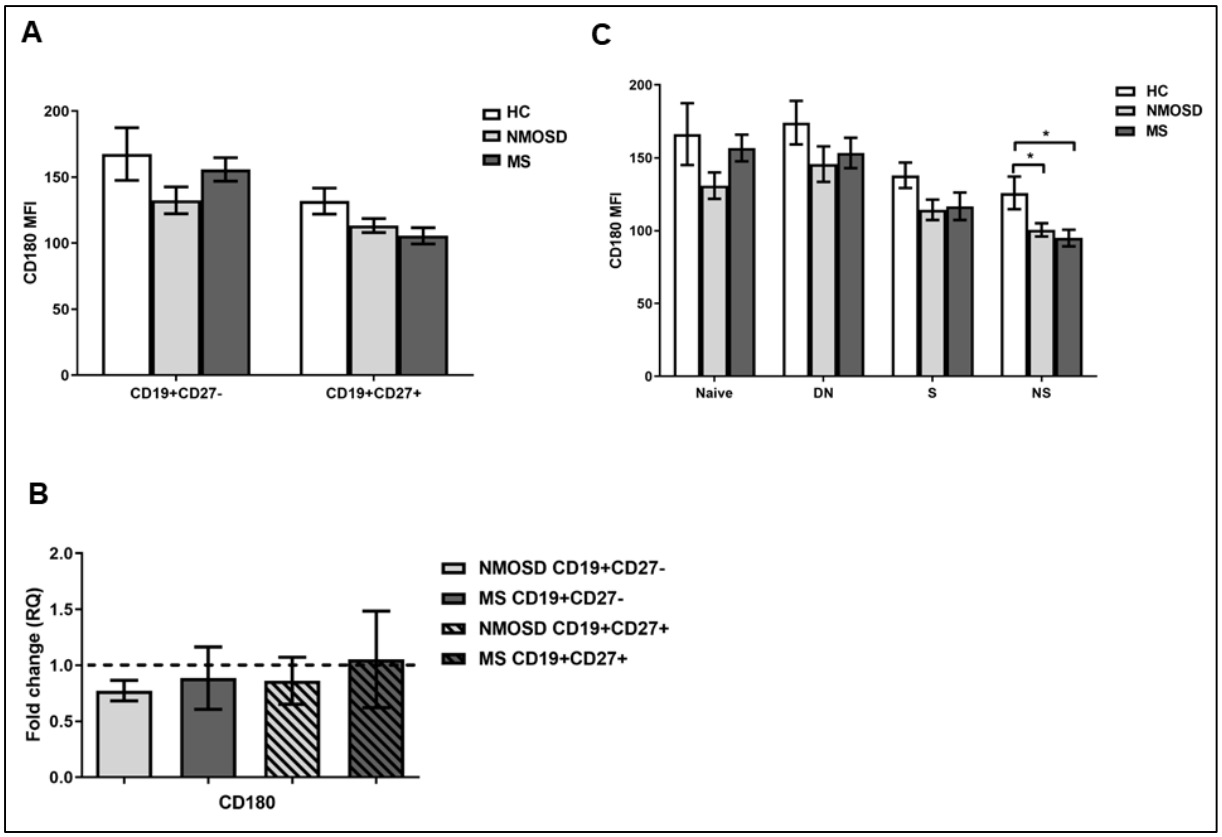


Figure 22. Analysis of CD180 expression in B cell subsets of neuromyelitis optica spectrum disorder (NMOSD) and multiple sclerosis (MS) patients. Flow cytometric analysis of CD180 expression in naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells in peripheral blood of NMOSD (*n* = 9), MS (*n* = 7) and healthy controls (HCs) (*n* = 5) (Panel A). CD180 mRNA expression in B cells of NMOSD (*n* = 5) and MS (*n* = 5) patients compared to HCs (*n* = 5). Gene expression was normalized to HCs and the horizontal line (value 1) represents the expression of control samples. Changes in gene expression are shown as relative quantification (RQ) values (Panel B). Flow cytometric analysis of CD180 expression in $CD19^+CD27^-IgD^+$ naïve, $CD19^+CD27^-IgD^-$ double negative (DN), $CD19^+CD27^+IgD^-$ switched memory (S) and $CD19^+CD27^+IgD^+$ non-switched memory (NS) B cells in peripheral blood of NMOSD (*n* = 9), MS (*n* = 7) and healthy controls (HCs) (*n* = 5) (Panel C). *Data are shown as mean ± standard error of the mean (SEM), * $p < 0.05$.*

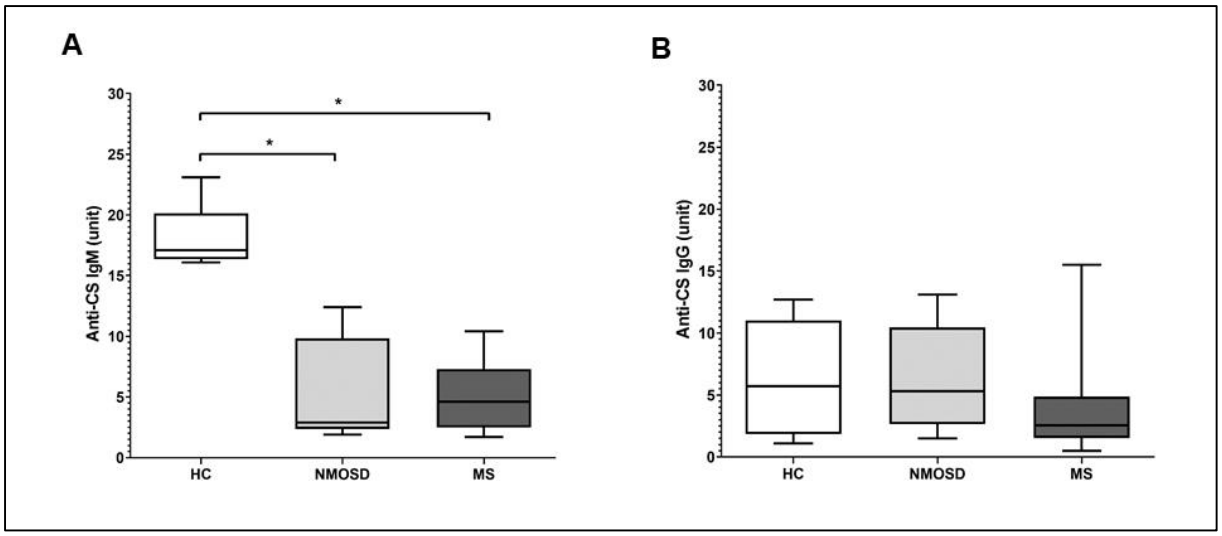


Figure 23. Natural autoantibody serum levels in neuromyelitis optica spectrum disorder (NMOSD) and multiple sclerosis (MS) patients. Anti-citrate synthase (CS) IgM (Panel A) and IgG (Panel B) levels in healthy controls (HCs), NMOSD and MS patients as measured by ELISA. *Boxes show interquartile ranges (IQR); whiskers indicate lowest and highest values; horizontal lines represent medians, n = 5 HC, n = 10 NMOSD, n = 13 MS, * $p < 0.05$.*

4.3.4 Increased anti-CS natural IgG autoantibody level in anti-Chlamydia Pneumoniae IgG-seropositive patients

Previous studies reported of associations between anti-bacterial antibodies and IgG natural autoantibodies in various autoimmune diseases (171, 172), and several infections, including Chlamydia pneumoniae, Chlamydia trachomatis, Mycoplasma pneumonia, Helicobacter pylori and Borrelia burgdorferi (148) are reported to have a potential role in the development of NMOSD or MS. Consequently, we measured IgM, IgG and IgA antibodies directed against these pathogens. We found that anti-Chlamydia pneumoniae IgG was detected in 54.5% (6/11) of NMOSD patients and in 14.3% (2/14) of MS patients. Anti-Mycoplasma pneumoniae IgG was detected in 9.1% (1/11) of NMOSD patients and in 21.4% (3/14) of MS patients. Anti-Helicobacter pylori IgG was detected in 18.2% (2/11) of NMOSD patients and in 14.3% (2/14) of MS patients. Neither of the NMOSD or MS patients was positive for anti-Chlamydia trachomatis IgG and anti-Borrelia burgdorferi IgG.

Table 20. Frequency of anti-bacterial IgM/G/A-seropositive cases in NMOSD and MS patients.

Infection	Autoantibody type	NMOSD (n=11)	MS (n=14)
Chlamydia pneumoniae	**IgM**	0	0
	IgG	6 (54.5%)	2 (14.3%)
	IgA	0	0
Chlamydia trachomatis	**IgM**	0	0
	IgG	0	0
	IgA	0	0
Mycoplasma pneumoniae	**IgM**	0	0
	IgG	1 (9.1%)	3 (21.4%)
	IgA	0	1 (7.1%)
Helicobacter pylori	**IgG**	2 (18.2%)	2 (14.3%)
	IgA	2 (18.2%)	0
Borrelia burgdorferi	**IgM**	0	1 (7.1%)
	IgG	0	0

Among the NMOSD and MS patients involved in the study, anti-Chlamydia pneumoniae IgG, anti-Mycoplasma pneumonia IgG and anti-Helicobacter pylori IgG seropositivity was the most frequent. Consequently, we analyzed the relationship between anti-CS IgM or IgG natural autoantibody levels and anti-bacterial antibody positivity in NMOSD and MS patients. We found higher tendency anti-CS IgG levels in the anti-Chlamydia pneumoniae IgG positive patients compared to the anti-Chlamydia pneumoniae IgG negative patients, but we did not find differences between neither the anti-Mycoplasma pneumonia IgG positive and the anti-Mycoplasma pneumonia IgG negative patients, nor the anti-Helicobacter pylori IgG positive and the anti-Helicobacter pylori IgG negative patients (Figure 24).

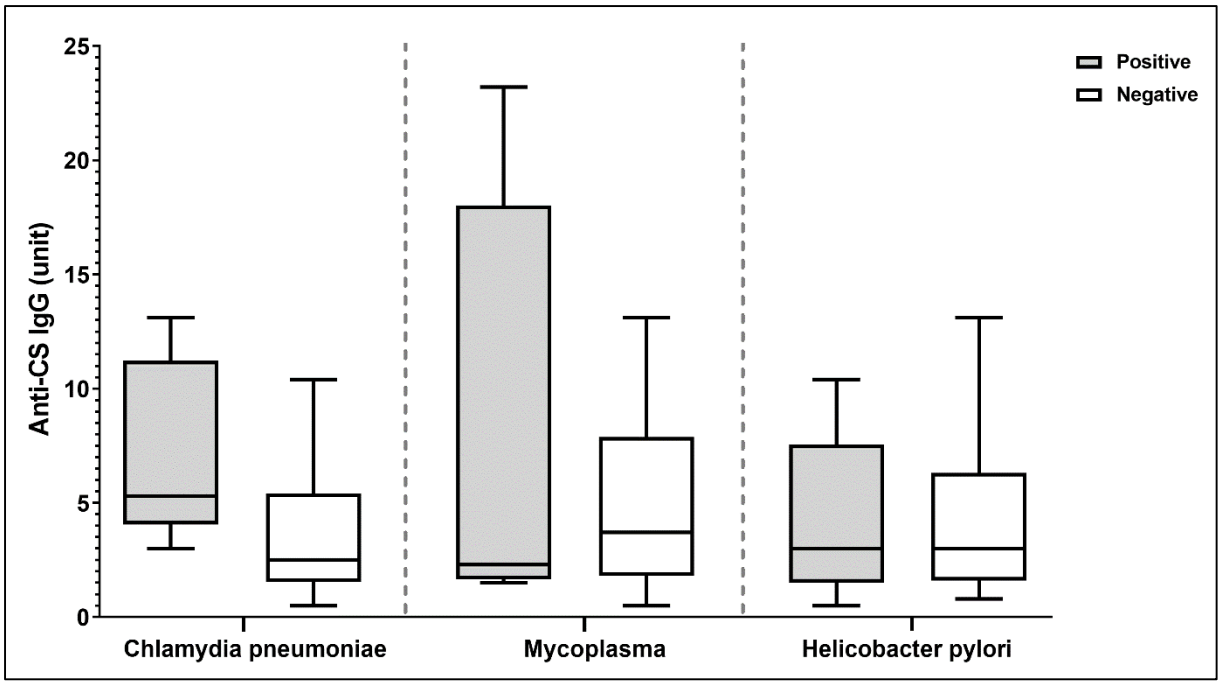


Figure 24. Natural IgG autoantibody levels in NMOSD and MS patients positive or negative for the investigated anti-bacterial IgG antibodies. Anti-CS IgG antibody levels in anti-Chlamydia pneumoniae, anti-Mycoplasma pneumonia and anti-Helicobacter pylori IgG positive and negative NMOSD and MS patients, as measured by ELISA. *Boxes show interquartile ranges (IQR); whiskers indicate lowest and highest values; horizontal lines represent medians,* $n_{NMOSD+MS} = 25$.

5. DISCUSSION

5.1 Single-center study of onconeural and neuronal cell surface autoantibody testing in Hungary

Due to the discovery of neuronal cell surface autoantibodies in the past decade, AE has been recognized as a distinct disease entity from onconeural autoantibody-associated PNSs (2, 187). The discovery of the characteristic autoantibodies in PNSs and AE has changed the diagnostic and therapeutic approaches to many neurological disorders previously considered to be idiopathic. In this study, we investigated the characteristics of autoantibody testing in patients with PNSs and AE, both diseases in which early and accurate clinical diagnosis plays a pivotal role. The retrospective analysis included laboratory test results from 2362 patients with suspected PNSs and 1034 patients with suspected AE, making it the most comprehensive study in Hungary to date. In the investigated period, from 2010 until 2018, the Department of Immunology and Biotechnology, Clinical Center, University of Pécs, Medical School, Pécs, Hungary was an exclusive center for carrying out onconeural and neuronal cell surface autoantibody testing in Hungary and received serum and/or CSF samples nationwide. Prevalence, incidence and age- and sex-based distribution of onconeural and neuronal cell surface autoantibodies represent data of the Hungarian population.

PNSs and AE are rare neurological disorders, and there is lack of convincing epidemiologic data. A recent study in Northeastern Italy reported of 0.89/100,000/year incidence in PNSs (188). In our study, we reported an overall 1.9/100,000/year incidence of onconeural autoantibody positivity in the Hungarian population. The higher incidence rate found in our cohort, might be due to that our study included all onconeural autoantibody positive cases, meanwhile, the cohort of Vogrig et al. (188) exclusively included data of patients with clinically confirmed PNSs. Previous studies reported that patients with PNSs tend to be of older age (e.g. anti-Hu-associated PNS, median age 63 years) (189), and the disease can affect adults of both sexes. Similarly, in our study, onconeural autoantibody positive patients had a median age of 62 years (range: 16-88 years), however, more females (*n=120*) than males (*n=70*) were affected. Similar to the study of Vogrig et al. (188), the three most frequently detected onconeural autoantibodies in our study were anti-Yo, anti-Hu and anti-Ma2 antibodies.

We reported an overall 0.6/100,000/year incidence of neuronal cell surface autoantibody positivity in the Hungarian population, which is similar to data previously reported

(0.8/100,000/year). Our data confirmed the relative prevalence of AE subtypes described previously (2). Anti-NMDAR autoantibodies were the first most frequent autoantibody type, the second most frequent was anti-LGI1, followed by anti-GABABR and anti-Caspr2, which is in agreement with previous reports (26, 30). Our data regarding the age and sex of AE patients agrees with the data published in the literature (173), anti-NMDAR autoantibodies mostly affected young females, anti-LGI1 antibodies most frequently occurred in middle-aged males, anti-GABABR autoantibodies occurred in elderly males, and anti-Caspr2 antibodies were present in male adults.

It is important to note that different methods are used for testing the autoantibodies in the two disease groups. Highly sensitive and specific multiplex cell-based assay is available for AE diagnostics, in which HEK293 cells expressing high levels of antigens of interest are used. For PNSs diagnostics, recombinant antigens are used in an immunoblot assay. Analysis of serum and CSF samples of patients with AE suggested that both types of samples should be tested, especially in patients with anti-NMDAR autoantibodies, since in most patients the autoantibodies are detected in the CSF, while the serum might be negative. However, in some anti-LGI1 antibody-positive patients, autoantibodies can be found only in serum or only in CSF (2). It has been reported previously that detection of the characteristic autoantibodies in clinically suspected AE could serve as confirmatory diagnosis, and in case of anti-NMDAR encephalitis, testing of CSF can be used to monitor disease activity, and autoantibody levels often correlate with patient outcome and relapse rates (1, 190). The highest sensitivity and specificity of the tests can be achieved by testing both serum and CSF. It should be noted, however, that false-positive results can occur more commonly when serum samples are tested. In contrast to AE, for the diagnosis of PNSs, testing the serum only is sufficient to detect the autoantibodies.

Our data are in agreement with previous reports on the frequency and distribution of onconeural and neuronal cell surface autoantibodies, detection of which can significantly aid the diagnosis of PNSs and AE, and also suggests treatment strategies. Early immunotherapy is often effective, and can reduce the severity of AE, promote recovery and decrease the risk of relapse (2, 173, 191, 192). Whereas, in PNSs, it is important to diagnose and treat the underlying tumour; immunotherapy has also been applied, however, it is rarely effective, although, in some patients, improvement of neurological symptoms has been reported (6, 15, 193, 194).

In conclusion, PNSs and AE are diverse groups of diseases, presenting with various clinical symptoms, tumour associations, and treatment responses. As the number of patients affected by these conditions is increasing and the spectrum of the newly identified autoantibodies broadens, it is important to employ reliable laboratory tests that allow accurate diagnosis to be made. The evaluation of patients with suspected AE should include testing for autoantibodies in both serum and CSF simultaneously, since some autoantibodies can be preferentially found only in serum or in CSF (2). In patients with PNSs, testing of serum alone might be adequate for establishment of precise diagnosis. Finally, early recognition of these diseases is important because without proper treatment they can have fatal outcome.

5.2 Clinical characteristics of neuronal cell surface autoantibody-mediated autoimmune encephalitis in a Hungarian cohort

We aimed to examine the characteristics of AE including clinical, laboratory, brain MRI features and outcome of AE in a Hungarian cohort. In accordance with previous publications of other series (22, 29, 30, 39), the most common AE type was anti-NMDAR encephalitis, followed by anti-LGI1 encephalitis (2, 26, 42). The study by Bien et al. (195) reported 576 antibody-positive patients during testing of 10,919 patients for a broad panel of neural antibodies, including onconeural and neuronal cell surface autoantibodies. Our previous study included results of 60 neuronal cell surface autoantibody positive patients (anti-NMDAR, anti-LGI1, anti-GABABR, anti-Caspr2), and this recent study exclusively included both serologically (neuronal cell surface antibody positive, but not onconeural autoantibody positive) and clinically positive definite AE patients. The distinct results in autoantibody frequencies detected in the two laboratories, may be due to the exclusive inclusion of neuronal cell surface autoantibody positive AE patients in our study, making it impossible to compare accurately the data of autoantibody frequencies. In our cohort, the sex ratio was close to equal, with anti-NMDAR encephalitis occurring slightly more frequently in men than women. Similar sex ratio was observed in a cohort of Chinese AE patients (176), although, published data claims that anti-NMDAR encephalitis affects predominantly young women (median age: 21 years) (2, 196). Dalmau et al (2) reported male predominance in anti-LGI1, anti-GABABR and anti-Caspr2 encephalitis, which was also observed in our cohort, although, the median age of our AE patients were 45-50 years compared to median values of 60-65 years in previous publications (2, 23).

In 70-86% of anti-NMDAR encephalitis patients, prodromal symptoms, such as headache, fever, nausea and vomiting were present (42, 44, 197). In our cohort, HSV infection occurred in one patient, who had secondary NMDAR encephalitis one month after HSV encephalitis. Previous publications (198) have reported HSV infection as being the most common viral trigger in AE patients and a possible trigger of anti-NMDAR encephalitis. The precise mechanism of HSV triggered AE is not clearly defined. Molecular mimicry or breakdown of immunological tolerance may be in the background (199). It has been stated that the most common symptoms of AE are psychiatric symptoms, seizures, involuntary movements, memory loss and sleep disorders (23). Besides these characteristic features, some rare manifestations, such as piloerection, also emphasized in a systematic review and related to anti-LGI1 encephalitis (54), cerebellar symptoms, neuropathy and skin rashes were also observed in our cohort. Previous publications have reported of FBDS as a typical symptom of anti-LGI1 encephalitis, often occurring a few weeks before onset of cognitive deficit in 26–71% of patients (26). In a retrospective study in the UK (200), 77% (20/26) of anti-LGI1 encephalitis patients experienced FBDS prior to the development of LE and found that early immunotherapy for FBDS might prevent progression to cognitive impairment. A prospective study of nine patients with anti-LGI1 encephalitis also revealed the beneficial effect of early immunotherapy (201). In our cohort of AE patients, FBDS was exclusively present in the LGI1 encephalitis patients with a lower prevalence (50%) compared to previous publications. Besides FBDS, hyponatraemia was also predominant in this group.

Examination of CSF has an important role in diagnosis, as the presence of pleocytosis is included in the diagnostic criteria for AE (174), although AE associated with autoantibodies against LGI1 and Caspr2 sometimes lack signs of inflammation in the CSF. The study of Hébert et al. (202) reported prevalence of CSF inflammatory markers, including elevated white blood cell count, elevated protein concentration and OCB in 95 patients with early active AE. Similar to our study, where 46% of AE patients had normal CSF results upon testing, in the cohort of Hébert et al. 44% of AE patients lacked CSF pleocytosis, 27% of patients, in addition to the lack of CSF pleocytosis, had normal protein concentration in the CSF and 14% of AE patients, besides the mentioned two CSF parameters, lacked OCB. In a retrospective study of anti-LGI1 encephalitis patients (53), CSF pleocytosis was identified in 23% of patients. Meanwhile, anti-NMDAR and anti-GABABR encephalitis are frequently associated with CSF inflammatory changes, such as pleocytosis and/or the presence of OCB (5, 203, 204). In a cohort of 100 patients with anti-NMDAR encephalitis (44) abnormal CSF findings were described in 95% of

patients, including CSF pleocytosis in 91%, increased total protein levels in 32% and OCB in 66.7% of anti-NMDAR patients. In a study analyzing clinical data of 44 anti-NMDAR encephalitis patients (205), reported CSF pleocytosis in 68% of patients, OCB was present in altogether 52% of patients during disease course. These findings were also observed in our study, where in anti-LGI1 and anti-Caspr2 positive patients, mainly normal CSF results were found, or increased total protein levels were detected in a few cases. In contrast with previous publications (5), CSF pleocytosis and OCB were exclusively detected in anti-NMDAR patients. Other abnormal CSF findings, such as elevated IgG index and/or increased total protein levels were detected in the anti-GABABR patient group. Data of prevalence of tumour association was also confirmed in our study, where 75% of AE patients presenting with tumour were anti-NMDAR and anti-GABABR positive. Ovarian teratoma is considered to be the most frequent tumour in anti-NMDAR encephalitis (39), but no association was found in our cohort. In a Chinese series, it was also rare and was only found in 2/72 patients (176). In anti-GABABR encephalitis, the dominant tumour type was SCLC, occurring in 66.7% of anti-GABABR positive patients, which is in agreement with the findings of Hermetter et al (39).

Most AE patients in our study showed favorable prognosis. In our cohort, the rate of complete recovery (66.7%) was slightly lower and death rate (10%) was mildly higher compared to data published by Deng et al (complete recovery: 81.3%, death rate: 6%) (176). In our cohort, relapses occurred exclusively among anti-NMDAR patients (1/19, 5.3% of anti-NMDAR patients). In a cohort of Chinese patients, relapses were also uncommon, occurring in 7/86 (8.1%) AE patients, mainly affecting NMDAR patients (5/72, 6.9% of anti-NMDAR patients) (176). However, in a cohort of Argentine AE patients, 25% of anti-NMDAR patients had relapses, but relapses also occurred in 25% of anti-LGI1 patients (178). Majority of the patients received first-line immunotherapy, steroid solely, or in combination with intravenous immunoglobulin or plasmapheresis, which had a good curative effect in most AE patients.

In 63.3% of AE patients in our cohort, signs of inflammation were detected in CSF and/or brain MRI, but no significant correlation was found between inflammatory markers and prognosis. In the study of Escudero et al. (185) a retrospective clinical analysis was conducted in 155 neuronal cell surface autoantibody positive patients with ≥ 60 years of age, but no brain MRI and CSF inflammatory changes were observed. In the cohort of Escudero et al. the most common autoantibody type was anti-LGI1 and the frequency of patients without evidence of CNS inflammation ranged from 25% (LGI1 antibodies) to 7% (GABABR antibodies). Escudero et al. reported higher frequency of non-inflammatory profile in anti-LGI1 patients ≥

60 years of age (25%), compared to younger patients (age < 60 years; 3%). In our study we also reported an overall significantly higher age of onset in AE patients presenting with inflammatory changes. Direct neuronal dysfunction caused by autoantibodies besides inflammatory infiltrates and BBB abnormalities may explain this observation (206). Previous publications have found association of CSF changes with worse outcome (45, 207), although early CSF and brain MRI abnormalities did not show strong correlation with disease outcome (208).

In conclusion, characteristics of AE in our Hungarian multicenter retrospective study are in agreement with previous findings. In addition, anti-NMDAR encephalitis patients presented with more severe disability at admission compared to anti-LGI1, anti-GABABR and anti-Caspr2 encephalitis. Presence of tumour was associated with worse outcome in AE patients compared to those patients without cancer. However, none of the anti-NMDAR encephalitis female patients had ovarian teratoma. 37% of patients lacked presence of both CSF inflammatory markers and brain MRI abnormalities. This observation, in addition to the role of auxiliary examinations (CSF analysis, EEG, brain MRI), emphasizes the importance of clinical presentation and autoantibody testing in diagnostic workflow. Our findings also highlight the significance of early introduction of first-line immunotherapy that resulted in favorable outcome in most AE patients in our cohort.

Our study is limited due to the retrospective data collection performed by clinicians using an online questionnaire, which may result in inadequate accuracy during reporting. The study design precludes the ability to address characteristics of AE that were not directly questioned or consistently recognized (for example, among sleep dysfunctions exclusively data regarding the presence of insomnia was collected). In our study, due to the low number of pediatric cases with age < 10 years (four cases), we could not confidently determine characteristics of pediatric patients. Although, the study has modest sample size, it summarizes detailed clinical data of 35 neuronal surface antibody positive patients from the 60 patients with positive autoantibody test results (n_{total} = 1,034 patients with suspected AE) from 2012 through 2018 in Hungary described in our previous study. Our data confirms results of previous publications and further clarifies clinical data of AE patients with neuronal cell surface autoantibodies.

5.3 Investigation of B cell abnormalities in patients with autoimmune CNS disorders

Studies focusing on the distribution of B cell subpopulations in NMOSD and MS are limited. Previous publications reported on analysis of peripheral blood naïve and memory B cell subsets in patients with NMOSD (78, 79, 209), however, most studies focus on regulatory B cells (210, 211). Several publications have investigated B cell subsets in different types and phases of MS (212, 213), these alterations still remained controversial, and most studies focused on peripheral blood B cell ratio changes following administration of disease modifying therapy (DMT) (209, 214, 215). In this study, we found no significant differences in the percentage of total $CD19^+$ B cells and distribution of B cell subsets in NMOSD or MS compared to HCs. This is in agreement with previous findings (109, 213, 216) reporting no significant differences in the distribution of naïve and memory B cell subsets in MS compared to HCs. However, decreased percentage of total $CD19^+$ B cells in RRMS patients (217) and increased proportion of memory B cells in untreated MS patients compared to HCs have also been reported (212, 218). Several studies reported of altered distribution of B cell subsets in MS patients treated with different disease modifying therapies (DMT). Increased proportion of memory B cells was described in MS patients treated with natalizumab (219) or atacicept (220), whereas, reduced proportion of memory B cells were reported in MS patients treated with dimethyl fumarate (221), interferon ß (222), glatiramer acetate (223), fingolimod (224) and alemtuzumab. Similar to our study, Habib et al. (213) did not observe any significant differences related to the type of disease modifying therapies (DMT) MS patients received. The effect of immunosuppressive therapies on alterations of B cell subsets in NMOSD and MS patients, have also been investigated. Janssen et al. (78) reported of significantly elevated levels of naïve B cell ratios in NMOSD compared to HC. Kowarik et al. (121) reported significantly elevated DN B cells and significantly lower memory B cells in NMOSD (*n=7*) compared to MS (*n=15*), and no significant differences in the proportion of NMOSD naïve B cells compared to MS and HCs. We also found significantly elevated ratio of DN B cells in NMOSD compared to MS, and no differences in the proportion of NMOSD naïve B cells compared to HCs, however, we found significantly increased frequencies of both S and NS memory B cells in NMOSD compared to MS.

The TLR homologue CD180 molecule activates the majority of B cells, resulting in phenotypic and functional alterations (225-227). Distinct expression and functions of CD180 on B cells have been associated with infection, chronic inflammation and autoimmune diseases (127, 136). Increased proportion of CD180-negative B cells was described in SLE (186) and

Sjögren's syndrome, and significantly decreased expression of CD180 in B cells of diffuse cutaneous systemic sclerosis (dcSSc) patients were reported (136). In this study, we found that the expression of CD180 was exclusively decreased in NS B cells in NMOSD and MS compared to HCs. It has already been described in SLE that the CD180-negative B cells are highly activated cells (186), and anti-CD180 antibody ligation resulted in decreased CD180 expression, thus the diminished CD180 expression of NS memory B cells in NMOSD and MS might be a result of B cell activation via CD180. NS B cells resemble B1 B cells (136, 228) and have innate-like features, suggesting their potential role in natural autoantibody production. The majority of natural autoantibodies are IgM isotype, polyreactive, low-titer antibodies, their presence in infants and their unaltered serum level during ≥ 5 years in adults indicates that these antibodies belong to the natural autoantibody repertoire established early in postnatal life (229, 230). They participate in removal of apoptotic cells, leading to decrease of inflammation, and also maintain tissue homeostatis, immunological balance and can prevent development of autoimmunity (146, 231). It was described that NS B cells are highly activated by CD180 ligation resulting in the enhancement of natural IgM autoantibody production (136). According to our results, diminished CD180 expression of NS B cells could contribute to lower anti-CS natural IgM levels found in NMOSD and MS compared to HCs. Our observation support the *in vivo* therapeutic efficacy of IVIGM (232), which was confirmed in experimental models of uveitis, myasthenia gravis, and MS (233, 234).

Correlation was reported between anti-CS natural IgG levels and cardiovascular-disease associated pathogens, including Chlamydia pneumoniae in coronary artery bypass grafting patients (171), and higher anti-CS natural IgG levels were detected in anti-measles IgG positive SLE patients (172), indicating a connection between natural IgG autoantibodies and infection-induced antibodies. Since data have been published on the possible involvement of Chlamydia pneumoniae, Chlamydia trachomatis, Mycoplasma pneumonia, Helicobacter pylori and Borrelia burgdorferi in the development of NMOSD or MS (148), we investigated the relationship between these anti-bacterial antibodies and natural autoantibodies. We found higher tendency of anti-CS natural IgG levels in anti-Chlamydia pneumoniae IgG positive NMOSD and MS patients than in anti-Chlamydia pneumoniae IgG negative patients. The titer of natural IgG autoantibodies fluctuates over time, they are abundant in human sera and their levels are influenced by age, gender and disease, indicating that their presence may be due to adaptive-like immune responses (142, 235).

Our results support the role of B cell subsets in the fine tuning of the immune homeostasis. We highlight the importance of natural autoantibodies, these first-line components

of the adaptive immune response in the balance of self-tolerance and anti-microbial immunity, and in the development of autoimmune diseases of the CNS.

5.4 Summary of the new scientific results

1. We report the first comprehensive report on national prevalence, incidence and age and sex-based distribution of onconeural and neuronal cell surface autoantibodies, and characteristics of AE patients diagnosed with neuronal cell surface antibody positivity (anti-NMDAR, anti-LGI1, anti-GABABR, anti-Caspr2) in Hungary to date.

2. We reported 1.9/100,000/year incidence of onconeural autoantibody positivity and 0.6/100,000/year incidence of neuronal cell surface autoantibody positivity in the Hungarian population. Onconeural autoantibodies, most commonly anti-Yo, anti-Hu and anti-Ma2 antibodies were detected in older individuals (median age: 62 years), and mainly occurred in females. Among neuronal cell surface autoantibodies: anti-NMDAR was the first most frequent in young females, anti-LGI1 was the second most frequent in middle-aged males, followed by anti-GABABR in elderly males, and anti-Caspr2 in male adults, similarly to other national data reported in the literature.

3. Our data show an increasing tendency in the number of onconeural and neuronal cell surface autoantibody positive patients, emphasizing the role of autoantibody testing in accurate diagnosis of PNSs and AE, which based on our data should be performed in both serum and CSF simultaneously.

4. Characteristics of neuronal cell surface autoantibody-mediated AE patients in the Hungarian cohort was similar to other previously reported national cohorts, however, differences were observed in anti-NMDAR encephalitis that showed no association with ovarian teratoma, occurred more frequently among young males, and presented with a more severe disability at the onset of the disease compared to anti-LGI1, anti-GABABR and anti-Caspr2 encephalitis. Besides, presence of tumour was associated with worse outcome in AE patients compared to those patients without cancer.

5. One-third of neuronal cell surface autoantibody-mediated AE patients in the Hungarian cohort lacked signs of inflammation both in CSF and brain MRI, which, in addition to the role of auxiliary examinations (CSF analysis, EEG, brain MRI), emphasizes the importance of

clinical symptoms and autoantibody testing in diagnostic workflow for early introduction of immunotherapy, which can lead to favorable outcome in AE patients.

6. Investigation of B cell distribution in autoimmune CNS disorders revealed significantly lower percentage of naïve and higher frequency of NS, S memory and DN peripheral blood B cells in NMOSD compared to MS.

7. Diminished CD180 expression of NS B cells was detected both in NMOSD and MS patients, which might be a result of B cell activation via CD180. We suggest that altered CD180 expression of NS B cells could contribute to the lower anti-CS IgM natural autoantibody production found in NMOSD and MS compared to HCs.

8. We reported significantly higher anti-CS natural IgG levels in anti-Chlamydia pneumoniae IgG positive NMOSD and MS patients than in anti-Chlamydia pneumoniae IgG negative patients, indicating the possible connection between natural IgG autoantibodies and infection-induced antibodies.

9. Our results suggest the role of B cell subsets in the fine tuning of the immune homeostasis. We highlight the importance of natural autoantibodies, these first-line components of the adaptive immune response in the balance of self-tolerance and anti-microbial immunity and in the development of autoimmune diseases of the CNS.

6. REFERENCES

1. Lee SK, Lee ST. The Laboratory Diagnosis of Autoimmune Encephalitis. J Epilepsy Res. 2016;6(2):45-50.
2. Dalmau J, Graus F. Antibody-Mediated Encephalitis. N Engl J Med. 2018;378(9):840-51.
3. Zuliani L, Graus F, Giometto B, Bien C, Vincent A. Central nervous system neuronal surface antibody associated syndromes: review and guidelines for recognition. J Neurol Neurosurg Psychiatry. 2012;83(6):638-45.
4. Graus F, Dalmau J. Paraneoplastic neurological syndromes in the era of immune-checkpoint inhibitors. Nat Rev Clin Oncol. 2019;16(9):535-48.
5. Blinder T, Lewerenz J. Cerebrospinal Fluid Findings in Patients With Autoimmune Encephalitis-A Systematic Analysis. Front Neurol. 2019;10:804.
6. Höftberger R, Rosenfeld MR, Dalmau J. Update on neurological paraneoplastic syndromes. Curr Opin Oncol. 2015;27(6):489-95.
7. Kannoth S. Paraneoplastic neurologic syndrome: A practical approach. Ann Indian Acad Neurol. 2012;15(1):6-12.
8. Chan AM, Baehring JM. Paraneoplastic neurological syndromes: a single institution 10-year case series. J Neurooncol. 2019;141(2):431-9.
9. Lorusso L, Precone V, Ferrari D, Ngonga GK, Russo AG, Paolacci S, et al. Paraneoplastic Neurological Syndromes: Study of Prevalence in a Province of the Lombardy Region, Italy. J Clin Med. 2020;9(10).
10. Oliveira V, Videira G, Samões R, Carneiro P, Neves E, Santos E. Paraneoplastic neurological syndromes with onconeural antibodies: A single center retrospective study. J Neurol Sci. 2020;418:117103.
11. Greenlee JE, Brashear HR. Antibodies to cerebellar Purkinje cells in patients with paraneoplastic cerebellar degeneration and ovarian carcinoma. Ann Neurol. 1983;14(6):609-13.
12. Bien CG, Vincent A, Barnett MH, Becker AJ, Blümcke I, Graus F, et al. Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis. Brain. 2012;135(Pt 5):1622-38.
13. Graus F, Delattre JY, Antoine JC, Dalmau J, Giometto B, Grisold W, et al. Recommended diagnostic criteria for paraneoplastic neurological syndromes. J Neurol Neurosurg Psychiatry. 2004;75(8):1135-40.
14. Grativvol RS, Cavalcante WCP, Castro LHM, Nitrini R, Simabukuro MM. Updates in the Diagnosis and Treatment of Paraneoplastic Neurologic Syndromes. Curr Oncol Rep. 2018;20(11):92.
15. Rosenfeld MR, Dalmau J. Paraneoplastic Neurologic Syndromes. Neurol Clin. 2018;36(3):675-85.
16. Rees JH. Paraneoplastic syndromes: when to suspect, how to confirm, and how to manage. J Neurol Neurosurg Psychiatry. 2004;75 Suppl 2:ii43-50.
17. Psimaras D, Carpentier AF, Rossi C, Euronetwork P. Cerebrospinal fluid study in paraneoplastic syndromes. J Neurol Neurosurg Psychiatry. 2010;81(1):42-5.
18. Gibbs E, Matsubara J, Cao S, Cui J, Forooghian F. Antigen-specificity of antiretinal antibodies in patients with noninfectious uveitis. Can J Ophthalmol. 2017;52(5):463-7.
19. Bataller L, Wade DF, Graus F, Stacey HD, Rosenfeld MR, Dalmau J. Antibodies to Zic4 in paraneoplastic neurologic disorders and small-cell lung cancer. Neurology. 2004;62(5):778-82.
20. Yamamoto AM, Gajdos P, Eymard B, Tranchant C, Warter JM, Gomez L, et al. Anti-titin antibodies in myasthenia gravis: tight association with thymoma and heterogeneity of nonthymoma patients. Arch Neurol. 2001;58(6):885-90.
21. Tschernatsch M, Singh P, Gross O, Gerriets T, Kneifel N, Probst C, et al. Anti-SOX1 antibodies in patients with paraneoplastic and non-paraneoplastic neuropathy. J Neuroimmunol. 2010;226(1-2):177-80.

22. Dalmau J, Geis C, Graus F. Autoantibodies to Synaptic Receptors and Neuronal Cell Surface Proteins in Autoimmune Diseases of the Central Nervous System. Physiol Rev. 2017;97(2):839-87.
23. Newman MP, Blum S, Wong RC, Scott JG, Prain K, Wilson RJ, et al. Autoimmune encephalitis. Intern Med J. 2016;46(2):148-57.
24. Honnorat J, Plazat LO. Autoimmune encephalitis and psychiatric disorders. Rev Neurol (Paris). 2018;174(4):228-36.
25. Fukata M, Yokoi N, Fukata Y. Neurobiology of autoimmune encephalitis. Curr Opin Neurobiol. 2018;48:1-8.
26. van Sonderen A, Petit-Pedrol M, Dalmau J, Titulaer MJ. The value of LGI1, Caspr2 and voltage-gated potassium channel antibodies in encephalitis. Nat Rev Neurol. 2017;13(5):290-301.
27. Celicanin M, Blaabjerg M, Maersk-Moller C, Beniczky S, Marner L, Thomsen C, et al. Autoimmune encephalitis associated with voltage-gated potassium channels-complex and leucine-rich glioma-inactivated 1 antibodies - a national cohort study. Eur J Neurol. 2017;24(8):999-1005.
28. Szots M, Blaabjerg M, Orsi G, Iversen P, Kondziella D, Madsen CG, et al. Global brain atrophy and metabolic dysfunction in LGI1 encephalitis: A prospective multimodal MRI study. J Neurol Sci. 2017;376:159-65.
29. Granerod J, Ambrose HE, Davies NW, Clewley JP, Walsh AL, Morgan D, et al. Causes of encephalitis and differences in their clinical presentations in England: a multicentre, population-based prospective study. Lancet Infect Dis. 2010;10(12):835-44.
30. Gable MS, Sheriff H, Dalmau J, Tilley DH, Glaser CA. The frequency of autoimmune N-methyl-D-aspartate receptor encephalitis surpasses that of individual viral etiologies in young individuals enrolled in the California Encephalitis Project. Clin Infect Dis. 2012;54(7):899-904.
31. Prüss H, Dalmau J, Harms L, Höltje M, Ahnert-Hilger G, Borowski K, et al. Retrospective analysis of NMDA receptor antibodies in encephalitis of unknown origin. Neurology. 2010;75(19):1735-9.
32. Lancaster E, Lai M, Peng X, Hughes E, Constantinescu R, Raizer J, et al. Antibodies to the GABA(B) receptor in limbic encephalitis with seizures: case series and characterisation of the antigen. Lancet Neurol. 2010;9(1):67-76.
33. Armangue T, Moris G, Cantarín-Extremera V, Conde CE, Rostasy K, Erro ME, et al. Autoimmune post-herpes simplex encephalitis of adults and teenagers. Neurology. 2015;85(20):1736-43.
34. Linnoila JJ, Binnicker MJ, Majed M, Klein CJ, McKeon A. CSF herpes virus and autoantibody profiles in the evaluation of encephalitis. Neurol Neuroimmunol Neuroinflamm. 2016;3(4):e245.
35. Hacohen Y, Deiva K, Pettingill P, Waters P, Siddiqui A, Chretien P, et al. N-methyl-D-aspartate receptor antibodies in post-herpes simplex virus encephalitis neurological relapse. Mov Disord. 2014;29(1):90-6.
36. Hughes EG, Peng X, Gleichman AJ, Lai M, Zhou L, Tsou R, et al. Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. J Neurosci. 2010;30(17):5866-75.
37. Kreye J, Wenke NK, Chayka M, Leubner J, Murugan R, Maier N, et al. Human cerebrospinal fluid monoclonal N-methyl-D-aspartate receptor autoantibodies are sufficient for encephalitis pathogenesis. Brain. 2016;139(Pt 10):2641-52.
38. Ohkawa T, Fukata Y, Yamasaki M, Miyazaki T, Yokoi N, Takashima H, et al. Autoantibodies to epilepsy-related LGI1 in limbic encephalitis neutralize LGI1-ADAM22 interaction and reduce synaptic AMPA receptors. J Neurosci. 2013;33(46):18161-74.
39. Hermetter C, Fazekas F, Hochmeister S. Systematic Review: Syndromes, Early Diagnosis, and Treatment in Autoimmune Encephalitis. Front Neurol. 2018;9:706.
40. Dutra LA, Abrantes F, Toso FF, Pedroso JL, Barsottini OGP, Hoftberger R. Autoimmune encephalitis: a review of diagnosis and treatment. Arq Neuropsiquiatr. 2018;76(1):41-9.
41. Dalmau J, Tüzün E, Wu HY, Masjuan J, Rossi JE, Voloschin A, et al. Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Ann Neurol. 2007;61(1):25-36.
42. Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. Lancet Neurol. 2011;10(1):63-74.

43. Titulaer MJ, McCracken L, Gabilondo I, Armangué T, Glaser C, Iizuka T, et al. Treatment and prognostic factors for long-term outcome in patients with anti-NMDA receptor encephalitis: an observational cohort study. Lancet Neurol. 2013;12(2):157-65.
44. Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, et al. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. Lancet Neurol. 2008;7(12):1091-8.
45. de Montmollin E, Demeret S, Brulé N, Conrad M, Dailler F, Lerolle N, et al. Anti-N-Methyl-d-Aspartate Receptor Encephalitis in Adult Patients Requiring Intensive Care. Am J Respir Crit Care Med. 2017;195(4):491-9.
46. Hart IK, Waters C, Vincent A, Newland C, Beeson D, Pongs O, et al. Autoantibodies detected to expressed K+ channels are implicated in neuromyotonia. Ann Neurol. 1997;41(2):238-46.
47. Kleopa KA, Elman LB, Lang B, Vincent A, Scherer SS. Neuromyotonia and limbic encephalitis sera target mature Shaker-type K+ channels: subunit specificity correlates with clinical manifestations. Brain. 2006;129(Pt 6):1570-84.
48. Irani SR, Alexander S, Waters P, Kleopa KA, Pettingill P, Zuliani L, et al. Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia. Brain. 2010;133(9):2734-48.
49. Lai M, Huijbers MG, Lancaster E, Graus F, Bataller L, Balice-Gordon R, et al. Investigation of LGI1 as the antigen in limbic encephalitis previously attributed to potassium channels: a case series. Lancet Neurol. 2010;9(8):776-85.
50. van Sonderen A, Schreurs MW, Wirtz PW, Sillevis Smitt PA, Titulaer MJ. From VGKC to LGI1 and Caspr2 encephalitis: The evolution of a disease entity over time. Autoimmun Rev. 2016;15(10):970-4.
51. Irani SR, Buckley C, Vincent A, Cockerell OC, Rudge P, Johnson MR, et al. Immunotherapy-responsive seizure-like episodes with potassium channel antibodies. Neurology. 2008;71(20):1647-8.
52. Paterson RW, Zandi MS, Armstrong R, Vincent A, Schott JM. Clinical relevance of positive voltage-gated potassium channel (VGKC)-complex antibodies: experience from a tertiary referral centre. J Neurol Neurosurg Psychiatry. 2014;85(6):625-30.
53. Ariño H, Armangué T, Petit-Pedrol M, Sabater L, Martinez-Hernandez E, Hara M, et al. Anti-LGI1-associated cognitive impairment: Presentation and long-term outcome. Neurology. 2016;87(8):759-65.
54. Tényi D, Bóné B, Horváth R, Komoly S, Illés Z, Beier CP, et al. Ictal piloerection is associated with high-grade glioma and autoimmune encephalitis-Results from a systematic review. Seizure. 2019;64:1-5.
55. Olsen AL, Lai Y, Dalmau J, Scherer SS, Lancaster E. Caspr2 autoantibodies target multiple epitopes. Neurol Neuroimmunol Neuroinflamm. 2015;2(4):e127.
56. Horresh I, Poliak S, Grant S, Bredt D, Rasband MN, Peles E. Multiple molecular interactions determine the clustering of Caspr2 and Kv1 channels in myelinated axons. J Neurosci. 2008;28(52):14213-22.
57. Lancaster E, Huijbers MG, Bar V, Boronat A, Wong A, Martinez-Hernandez E, et al. Investigations of caspr2, an autoantigen of encephalitis and neuromyotonia. Ann Neurol. 2011;69(2):303-11.
58. Irani SR, Pettingill P, Kleopa KA, Schiza N, Waters P, Mazia C, et al. Morvan syndrome: clinical and serological observations in 29 cases. Ann Neurol. 2012;72(2):241-55.
59. Liguori R, Vincent A, Clover L, Avoni P, Plazzi G, Cortelli P, et al. Morvan's syndrome: peripheral and central nervous system and cardiac involvement with antibodies to voltage-gated potassium channels. Brain. 2001;124(Pt 12):2417-26.
60. Terunuma M. Diversity of structure and function of GABA. Proc Jpn Acad Ser B Phys Biol Sci. 2018;94(10):390-411.
61. Lancaster E, Martinez-Hernandez E, Dalmau J. Encephalitis and antibodies to synaptic and neuronal cell surface proteins. Neurology. 2011;77(2):179-89.
62. Sprengel R. Role of AMPA receptors in synaptic plasticity. Cell Tissue Res. 2006;326(2):447-55.

63. Lai M, Hughes EG, Peng X, Zhou L, Gleichman AJ, Shu H, et al. AMPA receptor antibodies in limbic encephalitis alter synaptic receptor location. Ann Neurol. 2009;65(4):424-34.
64. Peng X, Hughes EG, Moscato EH, Parsons TD, Dalmau J, Balice-Gordon RJ. Cellular plasticity induced by anti-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor encephalitis antibodies. Ann Neurol. 2015;77(3):381-98.
65. Höftberger R, van Sonderen A, Leypoldt F, Houghton D, Geschwind M, Gelfand J, et al. Encephalitis and AMPA receptor antibodies: Novel findings in a case series of 22 patients. Neurology. 2015;84(24):2403-12.
66. Graus F, Boronat A, Xifró X, Boix M, Svigelj V, García A, et al. The expanding clinical profile of anti-AMPA receptor encephalitis. Neurology. 2010;74(10):857-9.
67. Hara M, Ariño H, Petit-Pedrol M, Sabater L, Titulaer MJ, Martinez-Hernandez E, et al. DPPX antibody-associated encephalitis: Main syndrome and antibody effects. Neurology. 2017;88(14):1340-8.
68. Boronat A, Gelfand JM, Gresa-Arribas N, Jeong HY, Walsh M, Roberts K, et al. Encephalitis and antibodies to dipeptidyl-peptidase-like protein-6, a subunit of Kv4.2 potassium channels. Ann Neurol. 2013;73(1):120-8.
69. Tobin WO, Lennon VA, Komorowski L, Probst C, Clardy SL, Aksamit AJ, et al. DPPX potassium channel antibody: frequency, clinical accompaniments, and outcomes in 20 patients. Neurology. 2014;83(20):1797-803.
70. Sabater L, Gaig C, Gelpi E, Bataller L, Lewerenz J, Torres-Vega E, et al. A novel non-rapid-eye movement and rapid-eye-movement parasomnia with sleep breathing disorder associated with antibodies to IgLON5: a case series, characterisation of the antigen, and post-mortem study. Lancet Neurol. 2014;13(6):575-86.
71. Nissen MS, Blaabjerg M. Anti-IgLON5 Disease: A Case With 11-Year Clinical Course and Review of the Literature. Front Neurol. 2019;10:1056.
72. Gaig C, Graus F, Compta Y, Högl B, Bataller L, Brüggemann N, et al. Clinical manifestations of the anti-IgLON5 disease. Neurology. 2017;88(18):1736-43.
73. Spatola M, Petit-Pedrol M, Simabukuro MM, Armangue T, Castro FJ, Barcelo Artigues MI, et al. Investigations in GABA. Neurology. 2017;88(11):1012-20.
74. Petit-Pedrol M, Armangue T, Peng X, Bataller L, Cellucci T, Davis R, et al. Encephalitis with refractory seizures, status epilepticus, and antibodies to the GABAA receptor: a case series, characterisation of the antigen, and analysis of the effects of antibodies. Lancet Neurol. 2014;13(3):276-86.
75. Ohkawa T, Satake S, Yokoi N, Miyazaki Y, Ohshita T, Sobue G, et al. Identification and characterization of GABA(A) receptor autoantibodies in autoimmune encephalitis. J Neurosci. 2014;34(24):8151-63.
76. Pettingill P, Kramer HB, Coebergh JA, Pettingill R, Maxwell S, Nibber A, et al. Antibodies to GABAA receptor α1 and γ2 subunits: clinical and serologic characterization. Neurology. 2015;84(12):1233-41.
77. Oh J, Levy M. Neuromyelitis optica: an antibody-mediated disorder of the central nervous system. Neurol Res Int. 2012;2012:460825.
78. Janssen M, Bruijstens AL, van Langelaar J, Wong Y, Wierenga-Wolf AF, Melief MJ, et al. Naive B cells in neuromyelitis optica spectrum disorders: impact of steroid use and relapses. Brain Commun. 2020;2(2):fcaa197.
79. Lebrun C, Cohen M, Rosenthal-Allieri MA, Bresch S, Benzaken S, Marignier R, et al. Only Follow-Up of Memory B Cells Helps Monitor Rituximab Administration to Patients with Neuromyelitis Optica Spectrum Disorders. Neurol Ther. 2018;7(2):373-83.
80. Li X, Wang L, Zhou L, ZhangBao J, Miao MZ, Lu C, et al. The imbalance between regulatory and memory B cells accompanied by an increased number of circulating T-follicular helper cells in MOG-antibody-associated demyelination. Mult Scler Relat Disord. 2019;36:101397.
81. Kessler RA, Mealy MA, Levy M. Treatment of Neuromyelitis Optica Spectrum Disorder: Acute, Preventive, and Symptomatic. Curr Treat Options Neurol. 2016;18(1):2.

82. Jarius S, Wildemann B. The history of neuromyelitis optica. J Neuroinflammation. 2013;10:8.
83. Lennon VA, Wingerchuk DM, Kryzer TJ, Pittock SJ, Lucchinetti CF, Fujihara K, et al. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. Lancet. 2004;364(9451):2106-12.
84. Papadopoulos MC, Verkman AS. Aquaporin 4 and neuromyelitis optica. Lancet Neurol. 2012;11(6):535-44.
85. Wingerchuk DM, Banwell B, Bennett JL, Cabre P, Carroll W, Chitnis T, et al. International consensus diagnostic criteria for neuromyelitis optica spectrum disorders. Neurology. 2015;85(2):177-89.
86. Jarius S, Ruprecht K, Kleiter I, Borisow N, Asgari N, Pitarokoili K, et al. MOG-IgG in NMO and related disorders: a multicenter study of 50 patients. Part 1: Frequency, syndrome specificity, influence of disease activity, long-term course, association with AQP4-IgG, and origin. J Neuroinflammation. 2016;13(1):279.
87. Jarius S, Ruprecht K, Kleiter I, Borisow N, Asgari N, Pitarokoili K, et al. MOG-IgG in NMO and related disorders: a multicenter study of 50 patients. Part 2: Epidemiology, clinical presentation, radiological and laboratory features, treatment responses, and long-term outcome. J Neuroinflammation. 2016;13(1):280.
88. Jarius S, Paul F, Weinshenker BG, Levy M, Kim HJ, Wildemann B. Neuromyelitis optica. Nat Rev Dis Primers. 2020;6(1):85.
89. Flanagan EP, Cabre P, Weinshenker BG, Sauver JS, Jacobson DJ, Majed M, et al. Epidemiology of aquaporin-4 autoimmunity and neuromyelitis optica spectrum. Ann Neurol. 2016;79(5):775-83.
90. Aboul-Enein F, Seifert-Held T, Mader S, Kuenz B, Lutterotti A, Rauschka H, et al. Neuromyelitis optica in Austria in 2011: to bridge the gap between neuroepidemiological research and practice in a study population of 8.4 million people. PLoS One. 2013;8(11):e79649.
91. Bukhari W, Prain KM, Waters P, Woodhall M, O'Gorman CM, Clarke L, et al. Incidence and prevalence of NMOSD in Australia and New Zealand. J Neurol Neurosurg Psychiatry. 2017;88(8):632-8.
92. Papp V, Iljicsov A, Rajda C, Magyari M, Koch-Henriksen N, Petersen T, et al. A population-based epidemiological study of neuromyelitis optica spectrum disorder in Hungary. Eur J Neurol. 2020;27(2):308-17.
93. Miyamoto K, Fujihara K, Kira JI, Kuriyama N, Matsui M, Tamakoshi A, et al. Nationwide epidemiological study of neuromyelitis optica in Japan. J Neurol Neurosurg Psychiatry. 2018;89(6):667-8.
94. Kim JE, Park SH, Han K, Kim HJ, Shin DW, Kim SM. Prevalence and incidence of neuromyelitis optica spectrum disorder and multiple sclerosis in Korea. Mult Scler. 2020;26(14):1837-44.
95. Jarius S, Ruprecht K, Wildemann B, Kuempfel T, Ringelstein M, Geis C, et al. Contrasting disease patterns in seropositive and seronegative neuromyelitis optica: A multicentre study of 175 patients. J Neuroinflammation. 2012;9:14.
96. Borisow N, Kleiter I, Gahlen A, Fischer K, Wernecke KD, Pache F, et al. Influence of female sex and fertile age on neuromyelitis optica spectrum disorders. Mult Scler. 2017;23(8):1092-103.
97. Quek AM, McKeon A, Lennon VA, Mandrekar JN, Iorio R, Jiao Y, et al. Effects of age and sex on aquaporin-4 autoimmunity. Arch Neurol. 2012;69(8):1039-43.
98. Eskandarieh S, Nedjat S, Abdollahpour I, Azimi AR, Moghadasi AN, Asgari N, et al. Environmental risk factors in neuromyelitis optica spectrum disorder: a case-control study. Acta Neurol Belg. 2018;118(2):277-87.
99. Messina S, Mariano R, Geraldes R, Kim SH, Satukijcha C, Vecchio D, et al. The influence of smoking on the pattern of disability and relapse risk in AQP4-positive Neuromyelitis Optica Spectrum Disorder, MOG-Ab Disease and Multiple Sclerosis. Mult Scler Relat Disord. 2021;49:102773.
100. Jitprapaikulsan J, Siritho S, Prayoonwiwat N. Vitamin D level status in Thai neuromyelitis optica patients. J Neuroimmunol. 2016;295-296:75-8.
101. Dobson R, Giovannoni G. Multiple sclerosis - a review. Eur J Neurol. 2019;26(1):27-40.

102. Nissimov N, Hajiyeva Z, Torke S, Grondey K, Brück W, Häusser-Kinzel S, et al. B cells reappear less mature and more activated after their anti-CD20-mediated depletion in multiple sclerosis. Proc Natl Acad Sci U S A. 2020;117(41):25690-9.
103. Häusler D, Häusser-Kinzel S, Feldmann L, Torke S, Lepennetier G, Bernard CCA, et al. Functional characterization of reappearing B cells after anti-CD20 treatment of CNS autoimmune disease. Proc Natl Acad Sci U S A. 2018;115(39):9773-8.
104. Kinzel S, Weber MS. B Cell-Directed Therapeutics in Multiple Sclerosis: Rationale and Clinical Evidence. CNS Drugs. 2016;30(12):1137-48.
105. Häusser-Kinzel S, Weber MS. The Role of B Cells and Antibodies in Multiple Sclerosis, Neuromyelitis Optica, and Related Disorders. Front Immunol. 2019;10:201.
106. Genç K, Dona DL, Reder AT. Increased CD80(+) B cells in active multiple sclerosis and reversal by interferon beta-1b therapy. J Clin Invest. 1997;99(11):2664-71.
107. Mathias A, Perriard G, Canales M, Soneson C, Delorenzi M, Schluep M, et al. Increased ex vivo antigen presentation profile of B cells in multiple sclerosis. Mult Scler. 2017;23(6):802-9.
108. Harp C, Lee J, Lambracht-Washington D, Cameron E, Olsen G, Frohman E, et al. Cerebrospinal fluid B cells from multiple sclerosis patients are subject to normal germinal center selection. J Neuroimmunol. 2007;183(1-2):189-99.
109. Harp CT, Ireland S, Davis LS, Remington G, Cassidy B, Cravens PD, et al. Memory B cells from a subset of treatment-naïve relapsing-remitting multiple sclerosis patients elicit CD4(+) T-cell proliferation and IFN-γ production in response to myelin basic protein and myelin oligodendrocyte glycoprotein. Eur J Immunol. 2010;40(10):2942-56.
110. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441(7090):235-8.
111. Korn T, Mitsdoerffer M, Croxford AL, Awasthi A, Dardalhon VA, Galileos G, et al. IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. Proc Natl Acad Sci U S A. 2008;105(47):18460-5.
112. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H, et al. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. J Immunol. 2007;178(10):6092-9.
113. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, et al. B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. J Exp Med. 2012;209(5):1001-10.
114. Li R, Rezk A, Miyazaki Y, Hilgenberg E, Touil H, Shen P, et al. Proinflammatory GM-CSF-producing B cells in multiple sclerosis and B cell depletion therapy. Sci Transl Med. 2015;7(310):310ra166.
115. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest. 2008;118(10):3420-30.
116. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood. 2011;117(2):530-41.
117. Link H, Huang YM. Oligoclonal bands in multiple sclerosis cerebrospinal fluid: an update on methodology and clinical usefulness. J Neuroimmunol. 2006;180(1-2):17-28.
118. Obermeier B, Mentele R, Malotka J, Kellermann J, Kümpfel T, Wekerle H, et al. Matching of oligoclonal immunoglobulin transcriptomes and proteomes of cerebrospinal fluid in multiple sclerosis. Nat Med. 2008;14(6):688-93.
119. Takahashi T, Fujihara K, Nakashima I, Misu T, Miyazawa I, Nakamura M, et al. Anti-aquaporin-4 antibody is involved in the pathogenesis of NMO: a study on antibody titre. Brain. 2007;130(Pt 5):1235-43.
120. Jarius S, Franciotta D, Paul F, Ruprecht K, Bergamaschi R, Rommer PS, et al. Cerebrospinal fluid antibodies to aquaporin-4 in neuromyelitis optica and related disorders: frequency, origin, and diagnostic relevance. J Neuroinflammation. 2010;7:52.

121. Kowarik MC, Astling D, Gasperi C, Wemlinger S, Schumann H, Dzieciatkowska M, et al. CNS Aquaporin-4-specific B cells connect with multiple B-cell compartments in neuromyelitis optica spectrum disorder. Ann Clin Transl Neurol. 2017;4(6):369-80.
122. Weber MS, Derfuss T, Brück W. Anti-Myelin Oligodendrocyte Glycoprotein Antibody-Associated Central Nervous System Demyelination-A Novel Disease Entity? JAMA Neurol. 2018;75(8):909-10.
123. Jurynczyk M, Messina S, Woodhall MR, Raza N, Everett R, Roca-Fernandez A, et al. Clinical presentation and prognosis in MOG-antibody disease: a UK study. Brain. 2017;140(12):3128-38.
124. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and functional features of central nervous system lymphatic vessels. Nature. 2015;523(7560):337-41.
125. Kowarik MC, Cepok S, Sellner J, Grummel V, Weber MS, Korn T, et al. CXCL13 is the major determinant for B cell recruitment to the CSF during neuroinflammation. J Neuroinflammation. 2012;9:93.
126. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
127. Schultz TE, Blumenthal A. The RP105/MD-1 complex: molecular signaling mechanisms and pathophysiological implications. J Leukoc Biol. 2017;101(1):183-92.
128. Divanovic S, Trompette A, Atabani SF, Madan R, Golenbock DT, Visintin A, et al. Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. Nat Immunol. 2005;6(6):571-8.
129. Koarada S, Tada Y, Ushiyama O, Morito F, Suzuki N, Ohta A, et al. B cells lacking RP105, a novel B cell antigen, in systemic lupus erythematosus. Arthritis Rheum. 1999;42(12):2593-600.
130. Kikuchi Y, Koarada S, Tada Y, Ushiyama O, Morito F, Suzuki N, et al. Difference in B cell activation between dermatomyositis and polymyositis: analysis of the expression of RP105 on peripheral blood B cells. Ann Rheum Dis. 2001;60(12):1137-40.
131. Kikuchi Y, Koarada S, Nakamura S, Yonemitsu N, Tada Y, Haruta Y, et al. Increase of RP105-lacking activated B cells in the peripheral blood and salivary glands in patients with Sjögren's syndrome. Clin Exp Rheumatol. 2008;26(1):5-12.
132. Fujita K, Akasaka Y, Kuwabara T, Wang B, Tanaka K, Kamata I, et al. Pathogenesis of lupus-like nephritis through autoimmune antibody produced by CD180-negative B lymphocytes in NZBWF1 mouse. Immunol Lett. 2012;144(1-2):1-6.
133. Kikuchi Y, Koarada S, Tada Y, Ushiyama O, Morito F, Suzuki N, et al. RP105-lacking B cells from lupus patients are responsible for the production of immunoglobulins and autoantibodies. Arthritis Rheum. 2002;46(12):3259-65.
134. Eriksen AB, Indrevær RL, Holm KL, Landskron J, Blomhoff HK. TLR9-signaling is required for turning retinoic acid into a potent stimulator of RP105 (CD180)-mediated proliferation and IgG synthesis in human memory B cells. Cell Immunol. 2012;279(1):87-95.
135. Yamazaki K, Yamazaki T, Taki S, Miyake K, Hayashi T, Ochs HD, et al. Potentiation of TLR9 responses for human naïve B-cell growth through RP105 signaling. Clin Immunol. 2010;135(1):125-36.
136. Erdő-Bonyár S, Rapp J, Minier T, Ráth G, Najbauer J, Czirják L, et al. Toll-Like Receptor Mediated Activation of Natural Autoantibody Producing B Cell Subpopulations in an Autoimmune Disease Model. Int J Mol Sci. 2019;20(24).
137. Palma J, Tokarz-Deptuła B, Deptuła J, Deptuła W. Natural antibodies - facts known and unknown. Cent Eur J Immunol. 2018;43(4):466-75.
138. Boyden SV. Natural antibodies and the immune response. Adv Immunol. 1966;5:1-28.
139. Grönwall C, Silverman GJ. Natural IgM: beneficial autoantibodies for the control of inflammatory and autoimmune disease. J Clin Immunol. 2014;34 Suppl 1:S12-21.
140. Lobo PI. Role of Natural Autoantibodies and Natural IgM Anti-Leucocyte Autoantibodies in Health and Disease. Front Immunol. 2016;7:198.
141. Savage HP, Baumgarth N. Characteristics of natural antibody-secreting cells. Ann N Y Acad Sci. 2015;1362:132-42.

142. Nagele EP, Han M, Acharya NK, DeMarshall C, Kosciuk MC, Nagele RG. Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. PLoS One. 2013;8(4):e60726.
143. Bournia VK, Vlachoyiannopoulos PG. Subgroups of Sjögren syndrome patients according to serological profiles. J Autoimmun. 2012;39(1-2):15-26.
144. Pisetsky DS. Antinuclear antibodies in rheumatic disease: a proposal for a function-based classification. Scand J Immunol. 2012;76(3):223-8.
145. Panda S, Ding JL. Natural antibodies bridge innate and adaptive immunity. J Immunol. 2015;194(1):13-20.
146. Maddur MS, Lacroix-Desmazes S, Dimitrov JD, Kazatchkine MD, Bayry J, Kaveri SV. Natural Antibodies: from First-Line Defense Against Pathogens to Perpetual Immune Homeostasis. Clin Rev Allergy Immunol. 2020;58(2):213-28.
147. Galeotti C, Kaveri SV, Bayry J. IVIG-mediated effector functions in autoimmune and inflammatory diseases. Int Immunol. 2017;29(11):491-8.
148. Cossu D, Yokoyama K, Hattori N. Bacteria-Host Interactions in Multiple Sclerosis. Front Microbiol. 2018;9:2966.
149. Sloane JA, Batt C, Ma Y, Harris ZM, Trapp B, Vartanian T. Hyaluronan blocks oligodendrocyte progenitor maturation and remyelination through TLR2. Proc Natl Acad Sci U S A. 2010;107(25):11555-60.
150. Kun D, Xiang-Lin C, Ming Z, Qi L. Chlamydia inhibit host cell apoptosis by inducing Bag-1 via the MAPK/ERK survival pathway. Apoptosis. 2013;18(9):1083-92.
151. Ivanova MV, Kolkova NI, Morgunova EY, Pashko YP, Zigangirova NA, Zakharova MN. Role of Chlamydia in Multiple Sclerosis. Bull Exp Biol Med. 2015;159(5):646-8.
152. Du C, Yao SY, Ljunggren-Rose A, Sriram S. Chlamydia pneumoniae infection of the central nervous system worsens experimental allergic encephalitis. J Exp Med. 2002;196(12):1639-44.
153. MacIntyre A, Hammond CJ, Little CS, Appelt DM, Balin BJ. Chlamydia pneumoniae infection alters the junctional complex proteins of human brain microvascular endothelial cells. FEMS Microbiol Lett. 2002;217(2):167-72.
154. Fainardi E, Castellazzi M, Seraceni S, Granieri E, Contini C. Under the microscope: focus on Chlamydia pneumoniae infection and multiple sclerosis. Curr Neurovasc Res. 2008;5(1):60-70.
155. Yoshimura S, Isobe N, Matsushita T, Yonekawa T, Masaki K, Sato S, et al. Distinct genetic and infectious profiles in Japanese neuromyelitis optica patients according to anti-aquaporin 4 antibody status. J Neurol Neurosurg Psychiatry. 2013;84(1):29-34.
156. Contini C, Seraceni S, Cultrera R, Castellazzi M, Granieri E, Fainardi E. Molecular detection of Parachlamydia-like organisms in cerebrospinal fluid of patients with multiple sclerosis. Mult Scler. 2008;14(4):564-6.
157. Waites KB, Talkington DF. Mycoplasma pneumoniae and its role as a human pathogen. Clin Microbiol Rev. 2004;17(4):697-728, table of contents.
158. Bahar M, Ashtari F, Aghaei M, Akbari M, Salari M, Ghalamkari S. Mycoplasma pneumonia seroposivity in Iranian patients with relapsing-remitting multipl sclerosis: a randomized case-control study. J Pak Med Assoc. 2012;62(3 Suppl 2):S6-8.
159. Casserly G, Barry T, Tourtellotte WW, Hogan EL. Absence of Mycoplasma-specific DNA sequence in brain, blood and CSF of patients with multiple sclerosis (MS): a study by PCR and real-time PCR. J Neurol Sci. 2007;253(1-2):48-52.
160. Lindsey J, Patel S. PCR for bacterial 16S ribosomal DNA in multiple sclerosis cerebrospinal fluid. Mult Scler. 2008;14(2):147-52.
161. Abramovitz P, Schvartzman P, Harel D, Lis I, Naot Y. Direct invasion of the central nervous system by Mycoplasma pneumoniae: a report of two cases. J Infect Dis. 1987;155(3):482-7.
162. Peek RM, Fiske C, Wilson KT. Role of innate immunity in Helicobacter pylori-induced gastric malignancy. Physiol Rev. 2010;90(3):831-58.

163. Jaruvongvanich V, Sanguankeo A, Jaruvongvanich S, Upala S. Association between Helicobacter pylori infection and multiple sclerosis: A systematic review and meta-analysis. Mult Scler Relat Disord. 2016;7:92-7.
164. Yao G, Wang P, Luo XD, Yu TM, Harris RA, Zhang XM. Meta-analysis of association between Helicobacter pylori infection and multiple sclerosis. Neurosci Lett. 2016;620:1-7.
165. Gavalas E, Kountouras J, Boziki M, Zavos C, Polyzos SA, Vlachaki E, et al. Relationship between. Ann Gastroenterol. 2015;28(3):353-6.
166. Efthymiou G, Dardiotis E, Liaskos C, Marou E, Tsimourtou V, Rigopoulou EI, et al. Immune responses against Helicobacter pylori-specific antigens differentiate relapsing remitting from secondary progressive multiple sclerosis. Sci Rep. 2017;7(1):7929.
167. Kountouras J, Zavos C, Polyzos SA, Deretzi G. The gut-brain axis: interactions between Helicobacter pylori and enteric and central nervous systems. Ann Gastroenterol. 2015;28(4):506.
168. Whiteside SK, Snook JP, Ma Y, Sonderegger FL, Fisher C, Petersen C, et al. IL-10 Deficiency Reveals a Role for TLR2-Dependent Bystander Activation of T Cells in Lyme Arthritis. J Immunol. 2018;200(4):1457-70.
169. Chmielewska-Badora J, Cisak E, Dutkiewicz J. Lyme borreliosis and multiple sclerosis: any connection? A seroepidemic study. Ann Agric Environ Med. 2000;7(2):141-3.
170. Schmutzhard E, Pohl P, Stanek G. Borrelia burgdorferi antibodies in patients with relapsing/remitting form and chronic progressive form of multiple sclerosis. J Neurol Neurosurg Psychiatry. 1988;51(9):1215-8.
171. Simon D, Gilicze O, Farkas N, Najbauer J, Németh P, Lénárd L, et al. Correlation of natural autoantibodies and cardiovascular disease-related anti-bacterial antibodies in pericardial fluid of cardiac surgery patients. Clin Exp Immunol. 2018;193(1):55-63.
172. Böröcz K, Simon D, Erdő-Bonyár S, Kovács KT, Tuba É, Czirják L, et al. Relationship between natural and infection-induced antibodies in systemic autoimmune diseases (SAD): SLE, SSc and RA. Clin Exp Immunol. 2021;203(1):32-40.
173. Ricken G, Schwaiger C, De Simoni D, Pichler V, Lang J, Glatter S, et al. Detection Methods for Autoantibodies in Suspected Autoimmune Encephalitis. Front Neurol. 2018;9:841.
174. Graus F, Titulaer MJ, Balu R, Benseler S, Bien CG, Cellucci T, et al. A clinical approach to diagnosis of autoimmune encephalitis. Lancet Neurol. 2016;15(4):391-404.
175. Lim JA, Lee ST, Moon J, Jun JS, Kim TJ, Shin YW, et al. Development of the clinical assessment scale in autoimmune encephalitis. Ann Neurol. 2019;85(3):352-8.
176. Deng S, Qiu K, Liu H, Wu X, Lei Q, Lu W. Clinical Characteristics and Short-Term Prognosis of Autoimmune Encephalitis: A Single-Center Cohort Study in Changsha, China. Front Neurol. 2019;10:539.
177. Nóbrega PR, Pitombeira MS, Mendes LS, Krueger MB, Santos CF, Morais NMM, et al. Clinical Features and Inflammatory Markers in Autoimmune Encephalitis Associated With Antibodies Against Neuronal Surface in Brazilian Patients. Front Neurol. 2019;10:472.
178. Melamud LI, Fernández VC, Manin A, Villa AM. Autoimmune encephalitis and immune therapy: lessons from Argentina. Acta Neurol Belg. 2020;120(3):565-72.
179. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol. 2018;17(2):162-73.
180. Filippi M, Bar-Or A, Piehl F, Preziosa P, Solari A, Vukusic S, et al. Multiple sclerosis. Nat Rev Dis Primers. 2018;4(1):43.
181. Tedder TF, Poe JC, Fujimoto M, Haas KM, Sato S. The CD19-CD21 signal transduction complex of B lymphocytes regulates the balance between health and autoimmune disease: systemic sclerosis as a model system. Curr Dir Autoimmun. 2005;8:55-90.
182. Agematsu K, Hokibara S, Nagumo H, Komiyama A. CD27: a memory B-cell marker. Immunol Today. 2000;21(5):204-6.
183. Gillinder L, Warren N, Hartel G, Dionisio S, O'Gorman C. EEG findings in NMDA encephalitis - A systematic review. Seizure. 2019;65:20-4.

184. Armangue T, Leypoldt F, Dalmau J. Autoimmune encephalitis as differential diagnosis of infectious encephalitis. Curr Opin Neurol. 2014;27(3):361-8.
185. Escudero D, Guasp M, Ariño H, Gaig C, Martínez-Hernández E, Dalmau J, et al. Antibody-associated CNS syndromes without signs of inflammation in the elderly. Neurology. 2017;89(14):1471-5.
186. Koarada S, Tada Y. RP105-negative B cells in systemic lupus erythematosus. Clin Dev Immunol. 2012;2012:259186.
187. Venkatesan A, Michael BD, Probasco JC, Geocadin RG, Solomon T. Acute encephalitis in immunocompetent adults. Lancet. 2019;393(10172):702-16.
188. Vogrig A, Gigli GL, Segatti S, Corazza E, Marini A, Bernardini A, et al. Epidemiology of paraneoplastic neurological syndromes: a population-based study. J Neurol. 2020;267(1):26-35.
189. Graus F, Keime-Guibert F, Reñe R, Benyahia B, Ribalta T, Ascaso C, et al. Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients. Brain. 2001;124(Pt 6):1138-48.
190. Wandinger KP, Leypoldt F, Junker R. Autoantibody-Mediated Encephalitis. Dtsch Arztebl Int. 2018;115(40):666-73.
191. Crisp SJ, Kullmann DM, Vincent A. Autoimmune synaptopathies. Nat Rev Neurosci. 2016;17(2):103-17.
192. Varley J, Taylor J, Irani SR. Autoantibody-mediated diseases of the CNS: Structure, dysfunction and therapy. Neuropharmacology. 2018;132:71-82.
193. de Beukelaar JW, Sillevis Smitt PA. Managing paraneoplastic neurological disorders. Oncologist. 2006;11(3):292-305.
194. Keime-Guibert F, Graus F, Fleury A, René R, Honnorat J, Broet P, et al. Treatment of paraneoplastic neurological syndromes with antineuronal antibodies (Anti-Hu, anti-Yo) with a combination of immunoglobulins, cyclophosphamide, and methylprednisolone. J Neurol Neurosurg Psychiatry. 2000;68(4):479-82.
195. Bien CG, Bien CI, Dogan Onugoren M, De Simoni D, Eigler V, Haensch CA, et al. Routine diagnostics for neural antibodies, clinical correlates, treatment and functional outcome. J Neurol. 2020;267(7):2101-14.
196. Hébert J, Riche B, Vogrig A, Muñiz-Castrillo S, Joubert B, Picard G, et al. Epidemiology of paraneoplastic neurologic syndromes and autoimmune encephalitides in France. Neurol Neuroimmunol Neuroinflamm. 2020;7(6).
197. Ma C, Wang C, Zhang Q, Lian Y. Emerging role of prodromal headache in patients with anti-N-methyl-D-aspartate receptor encephalitis. J Pain Res. 2019;12:519-26.
198. Armangue T, Spatola M, Vlagea A, Mattozzi S, Cárceles-Cordon M, Martinez-Heras E, et al. Frequency, symptoms, risk factors, and outcomes of autoimmune encephalitis after herpes simplex encephalitis: a prospective observational study and retrospective analysis. Lancet Neurol. 2018;17(9):760-72.
199. Leypoldt F, Titulaer MJ, Aguilar E, Walther J, Bönstrup M, Havemeister S, et al. Herpes simplex virus-1 encephalitis can trigger anti-NMDA receptor encephalitis: case report. Neurology. 2013;81(18):1637-9.
200. Irani SR, Michell AW, Lang B, Pettingill P, Waters P, Johnson MR, et al. Faciobrachial dystonic seizures precede Lgi1 antibody limbic encephalitis. Ann Neurol. 2011;69(5):892-900.
201. Irani SR, Stagg CJ, Schott JM, Rosenthal CR, Schneider SA, Pettingill P, et al. Faciobrachial dystonic seizures: the influence of immunotherapy on seizure control and prevention of cognitive impairment in a broadening phenotype. Brain. 2013;136(Pt 10):3151-62.
202. Hébert J, Gros P, Lapointe S, Amtashar FS, Steriade C, Maurice C, et al. Searching for autoimmune encephalitis: Beware of normal CSF. J Neuroimmunol. 2020;345:577285.
203. van Sonderen A, Thijs RD, Coenders EC, Jiskoot LC, Sanchez E, de Bruijn MA, et al. Anti-LGI1 encephalitis: Clinical syndrome and long-term follow-up. Neurology. 2016;87(14):1449-56.
204. van Sonderen A, Ariño H, Petit-Pedrol M, Leypoldt F, Körtvélyessy P, Wandinger KP, et al. The clinical spectrum of Caspr2 antibody-associated disease. Neurology. 2016;87(5):521-8.

205. Irani SR, Bera K, Waters P, Zuliani L, Maxwell S, Zandi MS, et al. N-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorder of both sexes. Brain. 2010;133(Pt 6):1655-67.
206. Lancaster E, Dalmau J. Neuronal autoantigens--pathogenesis, associated disorders and antibody testing. Nat Rev Neurol. 2012;8(7):380-90.
207. Wang W, Li JM, Hu FY, Wang R, Hong Z, He L, et al. Anti-NMDA receptor encephalitis: clinical characteristics, predictors of outcome and the knowledge gap in southwest China. Eur J Neurol. 2016;23(3):621-9.
208. Broadley J, Seneviratne U, Beech P, Buzzard K, Butzkueven H, O'Brien T, et al. Prognosticating autoimmune encephalitis: A systematic review. J Autoimmun. 2019;96:24-34.
209. Li R, Rezk A, Ghadiri M, Luessi F, Zipp F, Li H, et al. Dimethyl Fumarate Treatment Mediates an Anti-Inflammatory Shift in B Cell Subsets of Patients with Multiple Sclerosis. J Immunol. 2017;198(2):691-8.
210. Sun Y, Huang Y, Chen WW, Jia J, Wei T. The IL-10-producing regulatory B cells (B10 cells) and regulatory T cell subsets in neuromyelitis optica spectrum disorder. Neurol Sci. 2018;39(7):1307-8.
211. Han J, Sun L, Wang Z, Fan X, Wang L, Song YY, et al. Circulating regulatory B cell subsets in patients with neuromyelitis optica spectrum disorders. Neurol Sci. 2017;38(7):1205-12.
212. Jones AP, Kermode AG, Lucas RM, Carroll WM, Nolan D, Hart PH. Circulating immune cells in multiple sclerosis. Clin Exp Immunol. 2017;187(2):193-203.
213. Habib J, Deng J, Lava N, Tyor W, Galipeau J. Blood B Cell and Regulatory Subset Content in Multiple Sclerosis Patients. J Mult Scler (Foster City). 2015;2(2).
214. Kemmerer CL, Pernpeintner V, Ruschil C, Abdelhak A, Scholl M, Ziemann U, et al. Differential effects of disease modifying drugs on peripheral blood B cell subsets: A cross sectional study in multiple sclerosis patients treated with interferon-β, glatiramer acetate, dimethyl fumarate, fingolimod or natalizumab. PLoS One. 2020;15(7):e0235449.
215. Traub JW, Pellkofer HL, Grondey K, Seeger I, Rowold C, Brück W, et al. Natalizumab promotes activation and pro-inflammatory differentiation of peripheral B cells in multiple sclerosis patients. J Neuroinflammation. 2019;16(1):228.
216. Niino M, Hirotani M, Miyazaki Y, Sasaki H. Memory and naïve B-cell subsets in patients with multiple sclerosis. Neurosci Lett. 2009;464(1):74-8.
217. Haas J, Bekeredjian-Ding I, Milkova M, Balint B, Schwarz A, Korporal M, et al. B cells undergo unique compartmentalized redistribution in multiple sclerosis. J Autoimmun. 2011;37(4):289-99.
218. Dooley J, Pauwels I, Franckaert D, Smets I, Garcia-Perez JE, Hilven K, et al. Immunologic profiles of multiple sclerosis treatments reveal shared early B cell alterations. Neurol Neuroimmunol Neuroinflamm. 2016;3(4):e240.
219. Planas R, Jelčić I, Schippling S, Martin R, Sospedra M. Natalizumab treatment perturbs memory- and marginal zone-like B-cell homing in secondary lymphoid organs in multiple sclerosis. Eur J Immunol. 2012;42(3):790-8.
220. Sergott RC, Bennett JL, Rieckmann P, Montalban X, Mikol D, Freudensprung U, et al. ATON: results from a Phase II randomized trial of the B-cell-targeting agent atacicept in patients with optic neuritis. J Neurol Sci. 2015;351(1-2):174-8.
221. Lundy SK, Wu Q, Wang Q, Dowling CA, Taitano SH, Mao G, et al. Dimethyl fumarate treatment of relapsing-remitting multiple sclerosis influences B-cell subsets. Neurol Neuroimmunol Neuroinflamm. 2016;3(2):e211.
222. Rizzo F, Giacomini E, Mechelli R, Buscarinu MC, Salvetti M, Severa M, et al. Interferon-β therapy specifically reduces pathogenic memory B cells in multiple sclerosis patients by inducing a FAS-mediated apoptosis. Immunol Cell Biol. 2016;94(9):886-94.
223. Ireland SJ, Guzman AA, O'Brien DE, Hughes S, Greenberg B, Flores A, et al. The effect of glatiramer acetate therapy on functional properties of B cells from patients with relapsing-remitting multiple sclerosis. JAMA Neurol. 2014;71(11):1421-8.

224. Grützke B, Hucke S, Gross CC, Herold MV, Posevitz-Fejfar A, Wildemann BT, et al. Fingolimod treatment promotes regulatory phenotype and function of B cells. Ann Clin Transl Neurol. 2015;2(2):119-30.
225. Chaplin JW, Kasahara S, Clark EA, Ledbetter JA. Anti-CD180 (RP105) activates B cells to rapidly produce polyclonal Ig via a T cell and MyD88-independent pathway. J Immunol. 2011;187(8):4199-209.
226. Chaplin JW, Chappell CP, Clark EA. Targeting antigens to CD180 rapidly induces antigen-specific IgG, affinity maturation, and immunological memory. J Exp Med. 2013;210(10):2135-46.
227. Roe K, Shu GL, Draves KE, Giordano D, Pepper M, Clark EA. Targeting Antigens to CD180 but Not CD40 Programs Immature and Mature B Cell Subsets to Become Efficient APCs. J Immunol. 2019;203(7):1715-29.
228. Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, et al. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. Blood. 2004;104(12):3647-54.
229. Czömpöly T, Olasz K, Simon D, Nyárády Z, Pálinkás L, Czirják L, et al. A possible new bridge between innate and adaptive immunity: Are the anti-mitochondrial citrate synthase autoantibodies components of the natural antibody network? Mol Immunol. 2006;43(11):1761-8.
230. Czömpöly T, Olasz K, Nyárády Z, Simon D, Bovári J, Németh P. Detailed analyses of antibodies recognizing mitochondrial antigens suggest similar or identical mechanism for production of natural antibodies and natural autoantibodies. Autoimmun Rev. 2008;7(6):463-7.
231. Ehrenstein MR, Notley CA. The importance of natural IgM: scavenger, protector and regulator. Nat Rev Immunol. 2010;10(11):778-86.
232. Kaveri SV, Silverman GJ, Bayry J. Natural IgM in immune equilibrium and harnessing their therapeutic potential. J Immunol. 2012;188(3):939-45.
233. Warrington AE, Asakura K, Bieber AJ, Ciric B, Van Keulen V, Kaveri SV, et al. Human monoclonal antibodies reactive to oligodendrocytes promote remyelination in a model of multiple sclerosis. Proc Natl Acad Sci U S A. 2000;97(12):6820-5.
234. Bieber AJ, Warrington A, Asakura K, Ciric B, Kaveri SV, Pease LR, et al. Human antibodies accelerate the rate of remyelination following lysolecithin-induced demyelination in mice. Glia. 2002;37(3):241-9.
235. Tarlinton DM, McLean M, Nossal GJ. B1 and B2 cells differ in their potential to switch immunoglobulin isotype. Eur J Immunol. 1995;25(12):3388-93.

7. BIBLIOGRAPHY

Publications related to the thesis:

Böröcz, K., **Hayden, Z.**, Mészáros, V., Csizmadia, Z., Farkas, K., Kellermayer, Z., Balogh, P., Nagy, F., and Berki, T. (2018). "Autoimmune encephalitis: possibilities in the laboratory investigation." *Orv Hetil, 159*(3), 107-112. doi: 10.1556/650.2018.30951. Quartile Ranking: Q3 Impact Factor: 0.564 (2018)

Berki, T., **Hayden, Z.**, Böröcz, K., Csizmadia, Z., Kellermayer, Z., and Balogh, P. (2018). "Immunmediált kórképek diagnosztikája: laboratóriumi szakemberek és klinikusok együttműködése." *Neurológiai Praxis*, *1*(4), 14-15.

Hayden, Z., Böröcz, K., Csizmadia, Z., Balogh, P., Kellermayer, Z., and Berki, T. (2019). "A paraneoplásiás neurológiai szindrómák diagnosztikája és immunológiai vonatkozásai." *Magy Onkol, 63*(3), 261-267. Quartile Ranking: Q4 Impact Factor: 0.197 (2019)

Hayden, Z., Böröcz, K., Csizmadia, Z., Balogh, P., Kellermayer, Z., Bodó, K., Najbauer, J., and Berki, T. (2019). "Single-center study of autoimmune encephalitis-related autoantibody testing in Hungary." *Brain Behav*, *9*(12), e01454. doi: 10.1002/brb3.1454. Quartile Ranking: Q2 Impact Factor: 2.091 (2019)

Hayden, Z., Bóné, B., Orsi, G., Szots, M., Nagy, F., Csépány, T., Mezei, Z., Rajda, C., Simon, D., Najbauer, J., Illes, Z., and Berki, T. (2021). "Clinical characteristics and outcome of neuronal surface antibody-mediated autoimmune encephalitis patients in a national cohort." *Front. Neurol.* https://doi.org/10.3389/fneur.2021.611597 Quartile Ranking: Q2 Impact Factor: 2.889 (2020)

Hayden, Z., Erdő-Bonyár, S., Bóné, B., Balázs, N., Bodó, K., Illes, Z., Berki, T., and Simon, D. (2021). "Toll-like receptor homolog CD180 expression is diminished on natural autoantibody producing B cells of patients with autoimmune CNS disorders." *Journal of Immunology Research*. Accepted for publication. Quartile Ranking: Q1 Impact Factor: 3.327 (2020)

Publications not related to the thesis:

Orsi, G., Cseh, T., **Hayden, Z.**, Perlaki, G., Nagy, A. S., Giyab, O., Olsen, A. D., Madsen, S. J., Berki, T., and Illes, Z. (2021) "Microstructural and functional brain abnormalities in multiple sclerosis predicted by osteopontin and neurofilament light." *Mult Scler Relat Disord.* https://doi.org/10.1016/j.msard.2021.102923 Quartile Ranking: Q2 Impact Factor: 2.889 (2020)

Cummulative impact factor of publications related to the thesis: 9.068

Cummulative impact factor of all publications: 11.957

Topic related oral presentations:

Hayden Z., Böröcz K., and Berki T. „Autoimmun encephalitisek laboratóriumi diagnosztikája." DKK17 (Doktoranduszok a klinikai kutatásokban) Konferencia, Pécs, Magyarország, 2017.

Hayden Z., Böröcz K., and Berki T. „Autoimmune encephalitis: possibilities in the laboratory investigation." IDK 2018 (Interdiszciplináris Doktorandusz Konferencia), Pécs, Magyarország, 2018.

Berki T., Böröcz K., Csizmadia Z., **Hayden Z.**, and Balogh P. „Novel strategies to detect neurological ion-channel specific autoantibodies." MLDT (Magyar Laboratóriumi Diagnosztikai Társaság) 59. Naggyűlése, Pécs, Magyarország, 2018.

Hayden Z., Böröcz K., and Berki T. „New laboratory diagnostic methods in autoimmune encephalitis patients.” MedPÉCS 2018 (Medical Conference for PhD Students and Experts of Clinical Sciences) Konferencia, Pécs, Magyarország, 2018.

Hayden Z. „Az autoantitest meghatározás jelentősége az autoimmun encephalitis és a paraneoplasiás neurológiai szindróma differenciál diagnosztikájában.” III. Idegtudományi Centrum PhD és TDK Konferencia, Pécs, Magyarország, 2018. - PhD IV. section **prize for 2nd place**

Hayden Z. „Autoimmune encephalitis and paraneoplastic neurologic syndromes: importance of autoantibody detection in the differential diagnosis.” XVI. János Szentágothai Multidisciplinary Conference, Pécs, Magyarország, 2019. - Medical sciences section I. for PhD students **prize for 3rd place**

Hayden Z., Böröcz K., Csizmadia Z., Kellermayer Z., Balogh P., and Berki T. „Autoantitestek diagnosztikus jelentősége az idegrendszer autoimmun ioncsatorna betegségeiben.” 49. Membrántranszport Konferencia, Sümeg, Magyarország, 2019.

Hayden Z., Böröcz K., Csizmadia Z., Kellermayer Z., Balogh P., and Berki T. „A paraneopláziás neurológiai szindróma laboratóriumi diagnosztikájának egycentrumos retrospektív vizsgálata.” Magyar Neuroimmunológiai Társaság VI. Kongresszusa, Visegrád, Magyarország, 2019.

<u>Topic related Student Research oral presentations</u>

Hayden Z. „Transzfektált sejt alapú autoantitest meghatározási módszer az autoimmun encephalitisek laboratóriumi diagnosztikájában.” PTE ÁOK Tudományos Diákköri Konferencia, Pécs, Magyarország, 2016.

Hayden Z. „Új módszerek az autoimmun encephalitisek laboratóriumi diagnosztikájában.” PTE ÁOK Tudományos Diákköri Konferencia, Pécs, Magyarország, 2017. – Immunology section **prize for 2nd place**

Hayden Z. „Új módszerek az autoimmun encephalitisek laboratóriumi diagnosztikájában.” PTE ÁOK 11. Tudományos Diákköri Szalon, Pécs, Magyarország, 2017.

Hayden Z. „Új módszerek az autoimmun encephalitisek laboratóriumi diagnosztikájában.” 33. Országos Tudományos Diákköri Konferencia, Pécs, Magyarország, 2017.

Topic related poster presentations:

Hayden Z., Böröcz K., and Berki T. „ Statistical and epidemiological study of autoimmune encephalitis patients' samples in the past 6 years.” MLDT (Magyar Laboratóriumi Diagnosztikai Társaság) 59. Naggyűlése, Pécs, Magyarország, 2018.

Hayden Z., Böröcz K., and Berki T. “Statistical analysis of autoimmune encephalitis patients' samples.” MedPECS 2018 (Medical Conference for PhD Students and Experts of Clinical Sciences) Konferencia, Pécs, Magyarország, 2018.

Hayden Z., Böröcz K., Csizmadia Z., Kellermayer Z., Balogh P., and Berki T. „Single-center retrospective study of paraneoplastic neurologic syndrome-related autoantibody testing in Hungary.” MedPECS 2019 (Medical Conference for PhD Students and Experts of Clinical Sciences) Konferencia, Pécs, Magyarország, 2019.

Hayden Z., Böröcz K., Csizmadia Z., Kellermayer Z., Balogh P., and Berki T. „Autoantitestek diagnosztikus jelentősége az idegrendszer autoimmun ioncsatorna betegségeiben.” 49. Membrántranszport Konferencia, Sümeg, Magyarország, 2019. – **prize for poster presentation**

Hayden Z., Bóné B., Orsi G., Szots M., Nagy F., Csépány T., Mezei Z., Rajda C., Simon D., Najbauer J., Illes Z., and Berki T. „Clinical characteristics, treatment and outcome of anti-NMDAR encephalitis patients in Hungary: A multicenter retrospective study.” MedPECS 2020 (Medical Conference for PhD Students and Experts of Clinical Sciences) Konferencia, Pécs, Magyarország, 2020.

Hayden Z., Böröcz K., Csizmadia Z., Kellermayer Z., Balogh P., and Berki T. „Autoimmun encephalitis és paraneopláziás neurológiai szindróma pathomechanizmusának és laboratóriumi diagnosztikájának összehasonlító elemzése.” MIT (Magyar Immunológiai Társaság) Vándorgyűlés 2020. Online Konferencia, Magyarország, 2020.

8. ACKNOWLEDGEMENT AND FUNDING

8.1 Acknowledgement

I am grateful to my project leaders Prof. Timea Berki, M.D., Ph.D., Ds.C. and Beáta Bóné, M.D., Ph.D. for their leadership, support, advices and for providing me the opportunity to perform this project.

I wish to thank Prof. Zsolt Illés, M.D., Ph.D., Ds.C. for his support, advices and collaboration throughout the project.

I am also grateful to my colleagues and members of the Department of Immunology and Biotechnology, Medical School, University of Pécs for providing me technical facilities and for their kind help throughout the measurements during the course of the studies. I wish to thank József Najbauer, Ph.D., Ds.C. for his kind support and help in the preparation of the published studies. I am very greatful to Diána Simon, M.D., Ph.D. for her kind help in technical support, in the interpretation and statistical analysis of B cell activation studies. I would like to thank Noémi Balázs and Kornélia Bodó, Ph.D. their methodological support in B cell activation studies, and I wish to thank Szabolcs Fekete, Ph.D. for his technical support in creating the online questionnaire for data collection of Hungarian AE patients.

I am greatly thankful to Prof. József Janszky, M.D., Ph.D., Ds.C. and the Department of Neurology, Clinical Centre, University of Pécs for the collaboration. I express my special thanks to Erika Schleszák and Márti Attiláné Pál for supporting Beáta Bóné, M.D., Ph.D. and my work during patient involvement in the study.

I appreciate and thankful for the collaboration of Gergely Orsi, Ph.D., Mónika Szőts, M.D., Ph.D., Ferenc Nagy, M.D., Ph.D., Ds.C., Tünde Csépány, M.D., Ph.D., Ds.C., Zsolt Mezei, M.D., Ph.D. and Cecília Rajda, M.D., Ph.D., Ds.C.

I am very grateful to my whole family, especially to my parents and my couple for the patience and encouraging support.

8.2 Funding

This study was supported by Hungarian Grants: GINOP-232-15-2016-00050, EFOP 361-16-2016-00004 and Thematic Excellence Program 2020 - Institutional Excellence Sub-programme of the Ministry for Innovation and Technology in Hungary (the second thematic programme of the University of Pécs).

Az autoimmun encephalitisek laboratóriumi vizsgálati lehetőségei

**Böröcz Katalin[1] ▪ Hayden Zsófia dr.[1] ▪ Mészáros Viktória[1]
Csizmadia Zsuzsanna[1] ▪ Farkas Kornélia dr.[2] ▪ Kellermayer Zoltán dr.[1]
Balogh Péter dr.[1] ▪ Nagy Ferenc dr.[3] ▪ Berki Tímea dr.[1]**

**Pécsi Tudományegyetem, Általános Orvostudományi Kar, [1]Klinikai Központ, Immunológiai és Biotechnológiai Intézet, [2]Orvosi Statisztikai és Informatikai Tanszék, Pécs
[3]Somogy Megyei Kaposi Mór Oktatókórház, Kaposvár**

Bevezetés: Az elmúlt 10 évben a nem klasszifikálható neurológiai vagy pszichiátriai tünetegyüttes képében megjelenő encephalitisek esetén egyre gyakrabban igazolódik be, hogy a háttérben a központi idegrendszer valamely fehérjéje ellen induló autoimmun folyamat áll. A paraneoplasiás limbicus encephalitisek esetében intracelluláris antigének (anti-Hu/ANNA1, anti-Ri/ANNA2, anti-CV2/CRMP5 és anti-Ma2/Ta) ellen indul immunreakció, mely mögött tüdő-, ovarium- vagy heredaganat áll, és jellemző a rossz prognózis. Ezzel szemben az utóbbi években felfedezett, színes klinikai képpel megjelenő autoimmun encephalitisek mögött gyakran bizonyítható a neuronalis sejtfelszíni receptor (NMDAR, $GABA_BR$, AMPAR) vagy szinaptikus fehérje (LGI1, CASPR2) ellen képződő autoantitestek jelenléte, ami immunszuppressziós kezelésre jól reagál.
Célkitűzés: Célunk felhívni a figyelmet a neurológiai, pszichiátriai és intenzív terápiás ellátást igénylő autoimmun encephalitises esetek emelkedő számára, valamint az autoantitestek kimutatásának jelentőségére.
Módszer: Laboratóriumunkba az elmúlt 6 évben 836 autoimmun encephalitis irányú, 717 beteghez tartozó vizsgálatkérés érkezett. A betegek szérum- és liquormintáit 6 különböző receptorfehérjével transzfektált sejtvonalból álló BIOCHIP-en vizsgáltuk indirekt immunfluoreszcens technikával.
Eredmények: A vizsgált betegek 7,5%-ában tudtunk valamelyik receptorfehérje ellen IgG autoantitestet kimutatni. Gyakorisági sorrendben NMDAR > LGI1 > $GABA_BR$ > CASPR2 ellen találtunk pozitív eseteket.
Következtetés: Az autoantitest kimutatása segít a betegség korai stádiumban való felismerésében és a diagnózis felállításában. Mindez fontos, mert az időben felismert betegek eredményesen kezelhetőek plazmaferézissel vagy immunszuppresszív szerekkel, melyek hatékonyságát ismételt autoantitestmeghatározással lehet követni. Ezért a laboratóriumnak nagy szerepe lehet a gyorsan progrediáló kóros idegrendszeri folyamatok megállításában.
Orv Hetil. 2018; 159(3): 107–112.

Kulcsszavak: autoimmun encephalitis, autoantitest, neuronalis receptor, BIOCHIP

Autoimmune encephalitis: possibilities in the laboratory investigation

Introduction: The role of autoimmune responses against central nervous system (CNS) antigens in encephalitis presenting with non-classified neurologic or psychiatric symptoms has been appreciated in the past decade. Paraneoplastic limbic encephalitis has a poor prognosis and is most commonly associated with lung, ovarium, and testicular neoplasms, leading to immune reactions against intracellular antigens (anti-Hu/ANNA1, anti-Ri/ANNA2, anti-CV2/CRMP5 and anti-Ma2/Ta). In contrast, the recently described autoimmune encephalitis subtypes present with a broad spectrum of symptoms, respond to autoimmune therapies well and usually associate with autoantibodies against neuronal cell surface receptors (NMDAR, $GABA_BR$, AMPAR) or synaptic proteins (LGI1, CASPR2).
Aim: Our aim is to bring to awareness the increasing number of autoimmune encephalitis patients requiring neurologic, psychiatric and intensive care and to emphasize the significance of detecting various autoantibodies in diagnosing patients.
Method: In the past 6 years, our laboratory received 836 autoimmune encephalitis diagnostic test requests from a total of 717 patients. Serum and cerebrospinal fluid (CSF) samples were analysed with indirect immunofluorescence using a BIOCHIP consisting of cell lines transfected with 6 different receptor proteins.
Results: IgG autoantibodies against receptor proteins were present in 7.5% of patients. The frequency of positive samples was the following: NMDAR > LGI1 > $GABA_BR$ > CASPR2.

Conclusion: Detecting autoantibodies facilitates the diagnosis of autoimmune encephalitis in an early stage. Patients diagnosed early can be effectively treated with plasmapheresis and immunosuppressive drugs. The efficiency of therapies can be monitored by autoantibody detection. Therefore, the diagnostic immune laboratory plays an important role in proper diagnosis and in the prevention of rapidly progressing symptoms.

Keywords: autoimmune encephalitis, autoantibody, neuronal receptor, BIOCHIP

Böröcz K, Hayden Zs, Mészáros V, Csizmadia Zs, Farkas K, Kellermayer Z, Balogh P, Nagy F, Berki T. [Autoimmune encephalitis: possibilities in the laboratory investigation.] Orv Hetil. 2018; 159(3): 107–112.

(Beérkezett: 2017. szeptember 27.; elfogadva: 2017. október 18.)

Rövidítések

AMPAR1/2 = (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 1/2) α-amino-3-hidroxi-5-metil-4-izoxazolpropionsav-receptor-1/2; CASPR2 = (contactin-associated protein 2) kontaktinasszociált fehérje-2; CV2/CRMP5 = (collapsin response mediator protein 5) kollapszinreszponzív mediátorfehérje-5; FITC = (fluorescein isothiocyanate) fluoreszcein-izotiocianát; $GABA_BR$ = (gamma-aminobutyric acid receptor) gamma-aminovajsav-receptor; HEK293 = (human embryonic kidney cells) humán embrionális vesesejt; Hu/ANNA1 = (anti-neuronal nuclear antibody type I) 1-es típusú antineuronalis nukleáris antigén; IIF = (indirect immunofluorescence) indirekt immunfluoreszcencia; LGI1 = (leucine-rich, glioma-inactivated protein 1) leucinban gazdag gliomainaktivált fehérje-1; Ma2/Ta = (protein of the PNMA2 gene, paraneoplastic Ma Antigen 2/MM2) PNMA2-génhez kapcsolódó fehérje, MM2 onconeuralis antigén; NMDAR = (N-methyl-D-aspartate receptor) N-metil-D-aszpartát-receptor; Ri/ANNA2 = (antineuronal nuclear autoantibody type 2) 2-es típusú antineuronalis nukleáris antigén; SCLC = (small-cell lung cancer) kissejtes tüdőrák; VGKC = (voltage-gated potassium channel) feszültségfüggő káliumcsatorna

A klasszikus autoimmun mechanizmussal zajló perifériás ioncsatorna-betegségek mellett az utóbbi évtizedben leírtak központi idegrendszeri immunmediált ioncsatorna-betegségeket is. Ezeknek a kórképeknek a jelentőségét bizonyítja, hogy számuk egyre nőtt az elmúlt időszakban, ami elsősorban a megjelenő laboratóriumi diagnosztikai lehetőségek növekedésének köszönhető. Egyre gyakrabban igazolják laboratóriumi vizsgálatokkal, hogy a bizonytalan idegrendszeri tünetegyüttes mögött autoantitest mediálta immunreakció áll [1]. Az autoantitest megjelenése összefüggésbe hozható egy múltbéli infekcióval, vagy egy tumor által elindított onconeuralis ellenanyaggal asszociált paraneoplasiás folyamat, esetleg az immunrendszer egyensúlyának megbomlása áll a háttérben. Az autoantitestek hiánya nem zárja ki a patológiás folyamat autoimmun jellegét [1], mivel jelenleg nem rendelkezünk minden neuronalis receptor vagy ahhoz asszociált szinaptikus fehérjék ellen képződő autoantitest kimutatására alkalmas vizsgálati módszerrel.

Cikkünk célja felhívni a figyelmet az autoimmun encephalitises esetek emelkedő számára, valamint a szakszerűen elvégzett labordiagnosztikai vizsgálatok jelentőségére [2], hiszen a betegség korai stádiumban való felismerése hozzájárulhat a gyorsan progrediáló degeneratív idegrendszeri folyamatok diagnózisához, ezen keresztül a beteg életének megmentéséhez, ami az érintettek átlagéletkorát tekintve (jelentős a fiatalok [3], illetve középkorúak érintettsége) kiemelt jelentőségű. A jól karakterizált eredetű autoimmun encephalitiseket – etiológiájukat és patomechanizmusukat illetően – eddigi ismereteink alapján alapvetően két csoportba sorolhatjuk:

I. Paraneoplasiás jellegű, onconeuralis ellenanyaggal társult limbicus encephalitisek; elsősorban az anti-Hu, anti-Ri (ANNA-2) és az anti-Ta/Ma2, ritkábban az anti-CV2/CRMP5 antitest a tünetképző. A folyamat hátterét tumorok által expresszált ectopiás neuronalis antigének adják, melyek bemutatásra kerülnek a gyulladásos infiltrátumban lévő lymphocytáknak, ami keresztreakcióhoz vezet ezen neuronalis antigénekkel szemben. Mindennek eredményeként autoimmun reakció alakulhat ki az idegrendszerrel szemben, illetve ez a folyamat tovább progrediálhat a kiváltó tényezőként jelen lévő daganattól függetlenül [4]. A betegség prognózisa rossz, továbbá ezek az ellenanyagok egyéb „plusztüneteket” is okoznak a limbicus encephalitisre jellemző tünetek mellett [5].

II. Ezzel szemben a klasszikus autoimmun eredetű, ellenanyag-mediált limbicus encephalitisek prognózisa jóval ígéretesebb. A korábbi feltételezésekkel ellentétben ezek eredete nem egy fennálló infekció, hanem egy korábbi fertőzés, ritkábban okkult tumor következtében kialakult (molekuláris mimikri miatt létrejött) autoimmun válasz [1]. A komplex patomechanizmus ellenére ezeknek a betegeknek jók a kilátásaik, immunszuppresszióra, illetve plazmaferézisre jól reagálnak, az időben felismert degeneratív folyamatok visszafordíthatók [4]. Az Intézetünkben végzett, az autoimmun encephalitis kimutatását célzó laboratóriumi vizsgálatok ennek a betegcsoportnak az azonosítására irányulnak.

Autoimmun ellenanyag-mediált encephalitisek esetén az antitestek közvetlenül az ioncsatornákra vagy az ezekhez asszociált fehérjékre fejtik ki hatásukat, ami a csatornák diszfunkciójához vezet. Mivel ezek a fehérjék kulcsfontosságú szerepet játszanak a szinaptikus jelátvitelben és plaszticitásban, a hozzájuk társult autoimmunitás epi-

lepsziás görcsökben és neuropszichiátriai tünetekben nyilvánul meg. A különböző autoantitestek által kiváltott autoimmun encephalitisek klinikai manifesztációit illetően általánosságban jellemző az akut-szubakut neuropszichiátriai tünetek jelenléte, melyek gyors progressziót mutatnak. Kóros liquoreltérések jelentkezhetnek (emelkedett lymphocytaszám, oligoclonalis gamopathia). A koponya-MR-felvételeken a mediobasalis struktúrák érintettsége ábrázolódhat. Egyéb okok kizárása esetén (fennálló infekció, trauma, mérgezés, metabolikus ok, illetve sclerosis multiplex) autoimmun encephalitis irányában kell további vizsgálatokat indítani [6–10].

Laboratóriumi vizsgálatok nélkül is szembetűnőek a pszichiátriai betegséget utánzó tünetek, mint a kognitív zavarok, figyelemzavar, általános zavartság, hallucináció, hirtelen személyiségváltozás, illetve az epilepsziás rohamok jelenléte. További árulkodó jel lehet az érintett személyek viszonylag alacsony átlagéletkora (gyermekek, fiatalok, középkorúak), valamint, ha a kognitív zavarok motoros diszfunkciókkal is párosulnak, illetve a panaszok az idő előrehaladtával rapidan progrediálnak. Az autoimmun encephalitis egyes típusainak differenciálását segítő jellegzetes szimptómákat az *1. táblázat* mutatja be részletesen.

Az autoimmun encephalitisek napjainkban is sok esetben aluldiagnosztizáltak, hiszen a kórkép csak 2007-től vált ismertté, és a betegség hátterében gyakran más etiológiát feltételeznek. A betegségcsoport legismertebb és leggyakrabban előforduló képviselője az NMDAR-encephalitis, melyet az LGI1-, majd a $GABA_BR$-encephalitis követ [5, 11].

Módszer

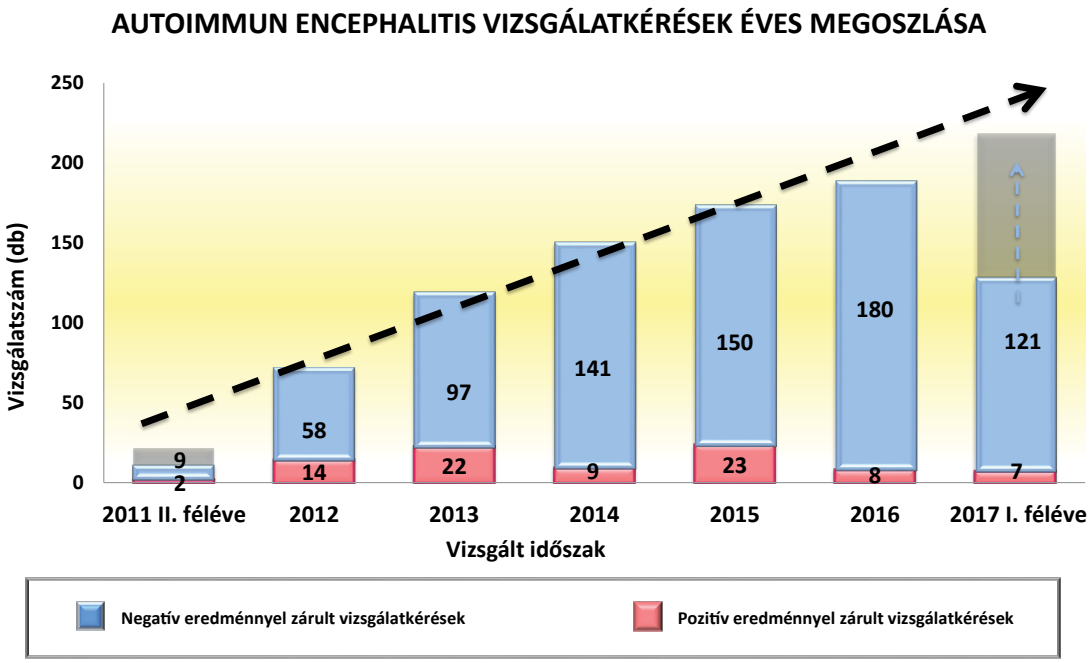
Egy retrospektív tanulmány keretein belül az Intézetünkben 2011 második félévétől napjainkig (2017. augusztus) végzett, sejtes alapú autoimmun encephalitis kimutatását célzó BIOCHIP-es indirekt immunfluoreszcencia (IIF)-vizsgálatok adatait dolgoztuk fel. Az eredmények statisztikai jellegű kiértékelése mellett adatainkat összevetettük a szakirodalommal, továbbá a vizsgálatkérések, valamint a pozitív minták volumenét és kvalitatív értékeit összegezve vizsgáltuk a betegség laboratóriumi diagnosztikájának jelen irányát.

Vizsgálatainkat modern, sejtalapú, nagy specificitású módszerrel végeztük (specificitás: NMDAR-liquor: 98,3%, NMDAR-szérum: 99,5%, CASPR2: 99,3%, $GABA_BR$, LGI1 és AMPAR1/2: 100%, szenzitivitás: NMDAR-liquor: 99,5%, NMDAR-szérum: 85,5%, $GABA_BR$: 82%, LGI1, CASPR2 és AMPAR1/2: 100%), amely 6 különböző receptorral vagy azzal asszociált fehérjével transzfektált HEK293 sejtvonalból összerakott BIOCHIP-eket tartalmaz (ún. Titerplane technika, EUROIMMUN kit). Az egyes altípusokra nézve eltérő lehet a liquor/szérum érzékenysége, így NMDAR-encephalitis esetén mindenképpen a liquort kell előnyben részesíteni a szérummal szemben, hiszen az előbbi esetén kevesebb az álnegativitás. A BIOCHIP a glutamátreceptorok (NMDAR, AMPAR1, AMPAR2 típusok), a CASPR2, az LGI1 fehérje és a $GABA_BR$ elleni IgG típusú autoantitestek kimutatására alkalmas IIF-val. A vizsgálatot a beteg szérumának vagy liquormintájának BIOCHIP-re történő felvitelével végezzük, amit előhí-

1. táblázat | A hat leggyakoribb receptor vagy azzal asszociált fehérje elleni autoantitest okozta autoimmun encephalitisek specifikus klinikai jellemzői

Az autoimmun encephalitis típusainak jellemzői [6]			
Receptorok/receptorasszociált fehérjék	A betegek jellemzői	Tumorasszociáció	Tünettan
NMDAR	A leggyakoribb típus. Bármely életkorban előfordulhat, de leánygyermekekben és fiatal nőkben gyakoribb	Ovarium/here teratoma	Influenzaszerű prodroma Kognitív és beszédzavarok Fluktuáló mentális állapot Kóros mozgásformák (perioralis dyskinesia) Komatózus állapot, légzésdepresszió
LGI1	Többségük férfi (≈65%), átlagéletkoruk 60 év	Ritkán: pajzsmirigyrák, SCLC, vesesejt-carcinoma, petefészek-teratoma, thymoma	Hyponatraemia Faciobrachialis dystoniás rohamok
CASPR2	Férfidominancia (≈80%)	Thymoma	A perifériás idegek hiperexcitabilitásával járó kórképekkel való társulás (Morvan-szindróma)
$GABA_BR$	Többségük férfi (férfi–nő arány: 55–45%), átlagéletkoruk ≈60 év	SCLC	Epilepsziás roham a bevezető tünet Ataxia és az opsoclonus-myoclonus
AMPAR1/2	Női dominancia (≈90%), bármely életkorban előfordulhat, a betegek átlagéletkora ≈60 év	Tüdő/emlő daganatok, thymoma	Limbicus encephalitisre/akut pszichózisra jellemző kezdet

AMPAR1/2 = (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor 1/2) α-amino-3-hidroxi-5-metil-4-izoxazolpropionsav-receptor-1/2; CASPR2 = (contactin-associated protein 2) kontaktinasszociált fehérje-2; $GABA_BR$ = (gamma-aminobutyric acid receptor) gamma-aminovajsav-receptor; LGI1 = (leucine-rich, glioma-inactivated protein 1) leucinban gazdag gliomainaktivált fehérje-1; NMDAR = (N-methyl-D-aspartate receptor) N-metil-D-aszpartát-receptor; SCLC = (small-cell lung cancer) kissejtes tüdőrák



1. ábra Az autoimmun encephalitis irányú vizsgálatkérések éves megoszlása

A vizsgálat bevezetése óta évente emelkedő számú minta érkezett laboratóriumunkba Magyarország neurológiai osztályairól. Ezen idő alatt összesen 717 beteghez tartozó 836 laboratóriumi vizsgálatkérés érkezett autoimmun encephalitis irányú vizsgálatra (egyazon kéréssel feladott szérumból és liquorból történő vizsgálat egy kérésnek számít). Az ismételt vizsgálatkérések miatt összesen 54 beteghez tartozó 85 pozitív mintát találtunk 6 év alatt. Az évente kiszűrt pozitív esetek száma nem korrelált az éves vizsgálatszámmal

vás követ antihumán IgG-FITC-vel jelölt másodlagos ellenanyaggal [12]. A műveletet szakorvos által végzett fluoreszcens mikroszkópos kiértékelés zárja.

Egy BIOCHIP hat sejttípusból összeállított mozaikot tartalmaz, amely hatfajta autoantitest kimutatására alkalmas. A módszer előnye, hogy egy mintából hat felszíni neuronalis antigén ellen termelődő antitest (NMDAR, AMPAR1, AMPAR2, CASPR2, LGI1, $GABA_BR$) kimutatására van lehetőség egyidejűleg. Pozitivitást egyszerre mindig csak egyfajta antitestre tapasztaltunk.

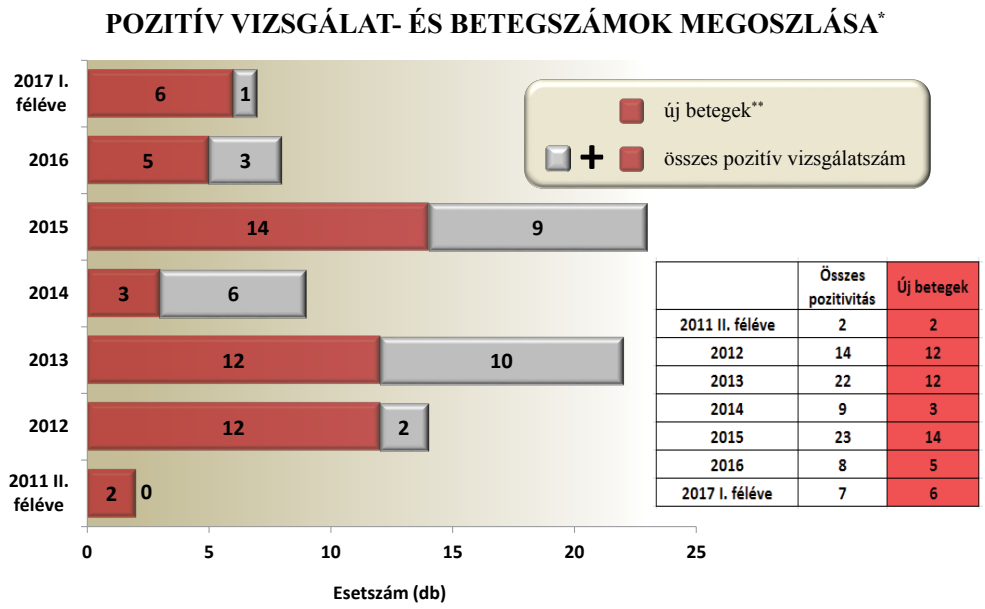
Eredmények

Intézetünkbe az elmúlt 6 évben összesen 836, szérumból, liquorból vagy mindkettőből kivitelezendő vizsgálatkérés érkezett, melyek 717 személyhez tartoztak. Ezek eredményeit dolgoztuk fel tanulmányunkban. Az összes elvégzett vizsgálatból 85 minta bizonyult pozitívnak, ezek 54 személyhez tartoztak.

Bár ritka betegségről van szó (a jóléti országokban a becsült incidencia 5–10 eset/100 000 fő/év [13, 14]), mind az éves vizsgálati volumen, mind a pozitív minták száma, nemkülönben a detektált specifikus autoantitestek köre a vizsgálat bevezetésétől fogva növekszik. Az éves bontású kimutatások tanúsága szerint évről évre fokozatosan fény derül újabb esetekre *(1. és 2. ábra)*, ami arra utal, hogy a betegség aluldiagnosztizáltsága a vizsgálat lehetőségének szélesebb körben való megismerésével csökkenhet. Az elmúlt hat évben az általunk célzottan vizsgált személyek 7,53%-a bizonyult pozitívnak. A BIOCHIP mozaikon szereplő hat antigénből csak négy ellen találtunk autoantitestet. Vezető helyen az NMDAR-pozitivitás áll (76%), ezt követi az LGI1- (13%), majd a $GABA_BR$- (9%) pozitivitás. CASPR2 ellenes autoantitestet egy betegnél találtunk *(3. ábra)*. Az általunk kapott eredmények a betegség előfordulását, valamint a betegek kor és nem szerinti megoszlását illetően a szakirodalommal egybehangzónak bizonyulnak *(1. táblázat)*.

Megbeszélés

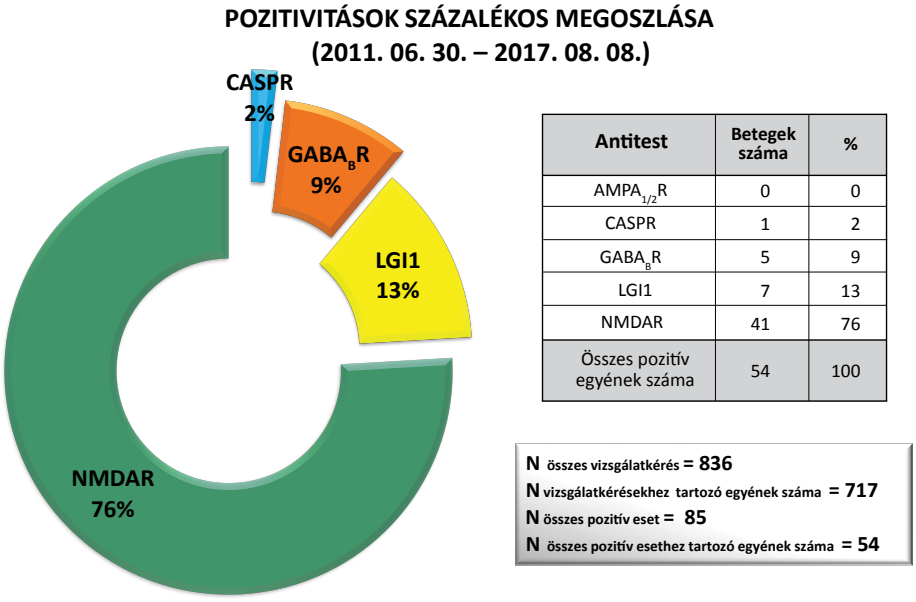
A klinikusok szempontjából nagy jelentősége van az autoimmun encephalitisek pontos laboratóriumi diagnosztikájának, hiszen a szerológiai vizsgálat autoimmun encephalitis gyanúja esetén megerősítheti a diagnózist, ezáltal az időben megkezdett terápia tünetmentessé teheti a betegeket, illetve segítségével a kezelésre adott válasz is megjósolható. A vizsgálat bevezetése óta az éves vizsgálatszámok növekedése figyelhető meg, ugyanakkor a kezdeti időszakkal összehasonlítva az utóbbi években csökkenni látszik a pozitív minták aránya. Ez feltételezésünk szerint abból adódhat, hogy az első időszakban a már klinikailag igazolt esetekben küldtek mintát a klinikusok a diagnózis megerősítésére, laboratóriumi vizsgálatra, míg a későbbi időszakban – a vizsgálati lehetőség elterjedésének köszönhetően – már differenciáldiagnosztikai céllal is kérik a vizsgálatot, kevésbé szelektált bete-



	Összes pozitivitás	Új betegek
2011 II. féléve	2	2
2012	14	12
2013	22	12
2014	9	3
2015	23	14
2016	8	5
2017 I. féléve	7	6

2. ábra A vizsgált időszakban az általunk használt BIOCHIP-en talált pozitív minták (piros és szürke terület együtt) és az ahhoz tartozó új autoimmun encephalitises betegek (piros) éves megoszlása

**Pozitív vizsgálatkérés: egy személyhez tartozó különböző mintaszámú kérések, melyek pozitív eredménnyel zárultak. Ugyanazon egyénnek több vizsgálatkérése is lehet (például terápia előtti és utáni állapot ellenőrzésére), akár egyazon éven belül*
***Új beteg: olyan személy, akinek mintája először adott pozitív eredményt. Ismétlés nélkül értendő egyének száma*



Antitest	Betegek száma	%
AMPA$_{1/2}$R	0	0
CASPR	1	2
GABA$_B$R	5	9
LGI1	7	13
NMDAR	41	76
Összes pozitív egyének száma	54	100

N összes vizsgálatkérés = 836
N vizsgálatkérésekhez tartozó egyének száma = 717
N összes pozitív eset = 85
N összes pozitív esethez tartozó egyének száma = 54

3. ábra A hat leggyakoribb receptor, illetve az azokhoz asszociált fehérjék ellen talált autoantitestek megoszlása (táblázat)

A sejtes alapú hat antigént tartalmazó BIOCHIP vizsgálati módszerrel 4 receptorfehérje ellen találtunk pozitív betegmintát. A leggyakoribb az NMDAR-pozitivitás (76%), ezt követi az LGI1- (13%), a GABA$_B$R- (9%) és a CASPR- (2%) pozitivitás. Eredményeink a betegség előfordulását, valamint a betegek kor és nem szerinti megoszlását illetően a szakirodalommal egybehangzónak bizonyultak

geknek is, az autoimmun encephalitis diagnózisának felállításához.

A betegség alacsony prevalenciája ellenére nem becsülhető alá a vizsgálat jelentősége, hiszen súlyos és potenciálisan halálos szindrómák fennállására utalhat. Paraneoplasiás eredet esetén a szindróma kialakulása akár évekkel megelőzheti a háttérben meghúzódó tumorra jellemző klinikai manifesztációkat. Ezáltal lehetőségünk van arra, hogy a malignitásokat időben felfedezzük, amikor még lehetséges a primer daganat gyógyítása. A be-

tegség ismerete nem paraneoplasiás eredet esetén is kiemelkedő jelentőségű, mert ebben az esetben az időben megkezdett immunmoduláló kezelés (szteroid, intravénás immunglobulin, plazmaferézis) az érintettek többségében gyógyulást eredményez. A későn megkezdett terápia esetén a válasz mértéke elmarad az időben megkezdett kezeléshez képest, ilyenkor a betegeknél súlyos maradványtünetek jelentkeznek, vagy akár halálos kimenetel is előfordulhat.

Következtetés

Az autoimmun encephalitis egyes típusai közül az NMDAR-encephalitis tekinthető a leggyakoribbnak, míg az előfordulási gyakoriságot illetően az LGI1-encephalitis áll a második helyen, ezt követi a $GABA_BR$-encephalitis [5]. Az NMDAR-encephalitis leánygyermekekben és fiatal nőkben fordul elő típusos esetben, míg az LGI1-, a $GABA_BR$- és a CASPR2-encephalitis a 60 év körüli középkorú férfiakban a leggyakoribb. Ezt saját beteganyagunk is alátámasztja. A vizsgálat kivitelezése és értékelése nagy szakértelmet és tapasztalatot igényel, ezért ezeknek a ritka betegségeknek a diagnosztikáját centrumokban kell végezni. Az eredmények mikroszkópos, szemikvantitatív kiértékelése ugyanis nem pusztán gyakorlott szakorvosi szemet feltételez, de a labor részéről is megfelelő kompetenciát kíván, mivel erre a betegségcsoportra fokozottan igaz, hogy a minta helyes megválasztása (bizonyos autoantitestek esetén a szérum, míg mások esetén a liquor érzékenyebb, de lehetőség szerint javasolt a kettő együttes vizsgálata) a talált pozitivitással összevetve határozza meg a definitív eredményt. Tapasztalataink szerint az idevonatkozó szakirodalmi irányelvek a gyakorlati úton megszerzett tudással egyesítve hivatottak garantálni az eredmények megbízhatóságát.

Anyagi támogatás: A dolgozat a GINOP-232-15-2016-00050, EFOP-361-16-2016-00004 pályázat segítségével készült.

Szerzői munkamegosztás: B. K.: A kísérletek értékelése, adatgyűjtés, statisztikai analízis, a közlemény megírása. H. Zs.: Laboratóriumi és klinikai adatok elemzése, irodalomkutatás, a közlemény megírása. M. V.: Szakmai tanácsadás. Cs. Zs.: A laboratóriumi tesztek elvégzése, szakmai ellenőrzése. F. K.: Statisztikai elemzés. K. Z.: A vizsgálatok értékelése. B. P.: A vizsgálatok értékelése. N. F.: A klinikai adatok elemzése, tanácsadás. B. T.: Témavezető, az adatok elemzése, a közlemény elkészítése. A cikk végleges változatát valamennyi szerző elolvasta és jóváhagyta.

Érdekeltségek: A szerzőknek nincsenek érdekeltségeik.

Irodalom

[1] Graus F, Titulaer MJ, Balu R, et al. A clinical approach to diagnosis of autoimmune encephalitis. Lancet Neurol. 2016; 15: 391–404.
[2] Koziolek MJ, Tampe D, Bähr M, et al. Immunoadsorption therapy in patients with multiple sclerosis with steroid-refractory optical neuritis. J Neuroinflammation 2012; 9: 80.
[3] Brenton JN, Goodkin HP. Antibody-mediated autoimmune encephalitis in childhood. Pediatr Neurol. 2016; 60: 13–23.
[4] Lancaster E. The diagnosis and treatment of autoimmune encephalitis. J Clin Neurol. 2016; 12: 1–13.
[5] Csépány T, Illés Zs. Clinical neuroimmunology. [Klinikai neuroimmunológia.] Mátyus–Benten Kiadó, Debrecen–Pécs, 2005. [Hungarian]
[6] Newman MP, Blum S, Wong RC, et al. Autoimmune encephalitis. Intern Med J. 2016; 46: 148–157.
[7] Irani SR, Alexander S, Waters P, et al. Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia. Brain 2010; 133: 2734–2748.
[8] Höftberger R, Titulaer MJ, Sabater L, et al. Encephalitis and $GABA_B$ receptor antibodies: Novel findings in a new case series of 20 patients. Neurology 2013; 81: 1500–1506.
[9] Lai M, Hughes EG, Peng X, et al. AMPA receptor antibodies in limbic encephalitis alter synaptic receptor location. Ann Neurol. 2009; 65: 424–434.
[10] Höftberger R, van Sonderen A, Leypoldt F, et al. Encephalitis and AMPA receptor antibodies: Novel findings in a case series of 22 patients. Neurology 2015; 84: 2403–2412.
[11] Khadilkar S, Soni G, Patil S, et al. Autoimmune encephalitis: An update. J Assoc Physicians India 2017; 65: 62–69.
[12] EUROIMMUN Medizinische Labordiagnostika AG. IIFT: Test instructions for the neuronal antigens profile 2 EUROLINE
[13] Vora NM, Holman RC, Mehal JM, et al. Burden of encephalitis-associated hospitalizations in the United States, 1998–2010. Neurology 2014; 82: 443–451.
[14] Jmor F, Emsley HC, Fischer M, et al. The incidence of acute encephalitis syndrome in Western industrialised and tropical countries. Virol J. 2008; 5: 134.

(Böröcz Katalin,
Pécs, Szigeti út 12., 7624
e-mail: borocz.katalin@pte.hu)

Laboratóriumi szakemberek és a klinikusok együttműködése

Immunmediált kórképek diagnosztikája

A központi idegrendszer immunmediált kórképeinél a klinikusok számára is fontos a specifikus autoantitestek detektálási módjának ismerete. A laboratóriumi tapasztalatokra alapozva lényeges, hogy az elküldött minta típusa (csak szérum vagy egyidejűleg szérum és liquor) megfelelő legyen. A vizsgálatkérések száma a tesztek bevezetése óta folyamatosan nő.

Laboratóriumunkban három ritka központi idegrendszert (KIR) érintő immunmediált kórképre, a neuromyelitis opticára (NMO), az autoimmun encephalitisekre (AE) és a paraneoplasiás neurológiai szindrómákra (PNS) jellemző autoantitestek kimutatását végezzük rutinszerűen. A három kórkép kialakulásáért eltérő patomechanizmus felelős.

Az NMO a központi idegrendszer súlyos, demyelinisatiós gyulladásos betegsége, mely a látóideget és a gerincvelőt egyaránt károsítja. NMO spektrumbetegségként (NMOSD) különítik el a térben limitált formáit, melyekben az említett két struktúra közül izoláltan az egyik érintett. A kórkép felismerésében a klinikai tünetek és képalkotó vizsgálatok mellett az aquaporin-4 vízcsatorna fehérje elleni (anti-AQP4) autoantitest kimutatásnak is döntő szerepe van (1). Az ellenanyag NMO-ban az esetek 80%-ában, míg NMOSD-ben az érintettek kisebb csoportjában detektálható (ismétlődő, relapszáló látóideg-gyulladásban vagy kétoldali látóideg-gyulladás esetén 5–27%-ban; kiterjedt lézióval járó transzverz myelitisben 14–55%-ban). Azon betegekben, akiknél anti-AQP4 autoantitest nem volt kimutatható, számos esetben myelin oligodendrocyta glikoprotein (MOG) ellenanyagot azonosítottak, mely szintén felelős lehet a kórkép kialakulásáért (2). NMO-ban egy korábbi infekció által kiváltott specifikus B-sejt-aktiváció vezet a központi idegrendszer fő vízcsatornája, az AQP4 elleni autoantitestek képződéséhez és IgG- vagy IgM-lerakódáshoz a központi idegrendszerben. Az ellenanyagok elsősorban az AQP4-et legnagyobb mennyiségben expresszáló astrocytákat károsítják, azonban a gyulladás az oligodendrocytákat is érinti és demyelinisatiót okoz.

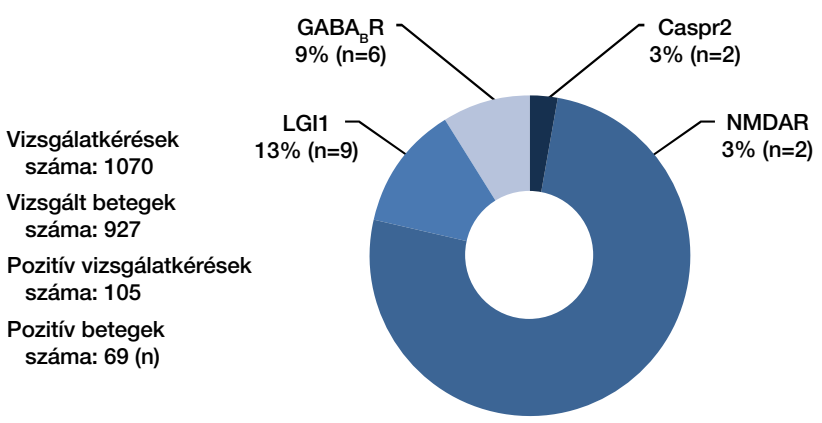
Az AE a neuronális szinapszisok receptorfehérjéi ellen termelődő autoantitestek hatására kialakuló neurológiai kórkép, mely limbikus encephalitis formájában (neuropszichiátriai tünetek, pszichózis, memória-, tudat- és viselkedési zavarok, epilepsziás görcsök) manifesztálódik az érintett betegekben. A klinikai tünettan és képalkotó vizsgálatok (koponya-MRI: a mediotemporális lebenyek területén jelentkező FLAIR/T2 hiperintenzitás), valamint kóros liquoreltérések (emelkedett limfocitaszám, oligoklonális gammopátia) mellett, az AE-re jellemző autoantitestek detektálása is fontos része a diagnosztikának (3). AE esetén az autoantitestek a neuronális sejtfelszíni receptorok (NMDAR, GABABR, AMPAR1, AMPAR2), illetve ezek asszociált fehérjéi (LGI1, Caspr2) ellen képződnek. Az ellenanyagok a célantigének extracelluláris epitópjához kötődve megváltoztatják azok szerkezetét és funkcióját, és reverzibilis neuronkárosodáshoz vezetnek (4). A különböző autoantitestek eltérő mechanizmussal okoznak betegséget: anti-NMDAR encephalitisben a receptor internalizáció, anti-LGI1 encephalitisben a fehérje interakció gátlása és anti-GABABR encephalitisben a célantigén funkcionális gátlása által fejtik ki hatásukat (5, 6).

A PNS betegek klinikai tünettana rendkívül változatos, hiszen a kórkép egyaránt érintheti a centrális vagy a perifériás idegrendszer bármely részét, beleértve a retinát és a vázizmokat. Az érintettek többségében a szindróma egyidejűleg gyakran társul különböző típusú tumorokkal (SCLC, ovárium-, emlőtumor, thymoma). A neurológiai tünetek megelőzik a daganat észlelését, így a PNS-re jellemző onkoneurális autoantitestek segíthetik a tumor korai felismerését, amely fontos szerepet játszik a betegek életkilátásának javításában (7). PNS-ben a tumorok által expresszált neuronális fehérjék jelenléte váltja ki az autoantitest-termelést, mely intracelluláris fehérjék (Ma2, Yo, CV2, Hu, amphiphysin, Ri, Tr, GAD65, Zic4, titin, SOX1és recoverin) ellen irányul. A képződő onkoneurális ellenanyagok valószínűleg nem patogének (inkább a betegség és az egyidejűleg fennálló tumor markereként szolgálhatnak a diagnózis során), és döntően T-sejt-mediálta folyamatok vezetnek az irreverzibilis neuronkárosodáshoz (3). Az AE és a PNS klinikai megjelenése hasonló, azonban az eltérő patomechanizmusból adódóan (AE-ben reverzibilis, míg PNS-ben irreverzibilis neuronkárosodás) az érintett betegek kezelése és terápiára adott válasza eltér. AE betegeknél az immunterápia (pl. szteroidok, plazmaferezis, immunszuppresszió, IVIG) az esetek döntő többségében javulást eredményez a tünetekért felelős autoantitestek szintjének csökkentése által. PNS-ben az irreverzibilis károsodásból kifolyólag az immunterápia ritkán hatásos, azonban az időben megkezdett tumorkezelés stabilizálhatja a betegek állapotát (8).

Az NMO, az AE és a PNS akár fatális kimenetelűek is lehetnek, így az alacsony prevalencia ellenére nagy jelentőséggel bír a megfelelő terápia megválasztása, mely a gyors és pontos diagnózison alapul. Ennek elengedhetetlen részét képezik az autoantitest-detektáláson alapuló labordiagnosztikai módszerek.

Az NMO, az AE és a PNS klinikai jellemzőit, valamint a jellegzetes autoantitesteket és ezek detektálására szolgáló labordiagnosztikai módszereket az *1. táblázatban* foglaltuk össze.

1.A ábra: Az autoimmun encephalitis irányú vizsgálatkérések feldolgozása során kapott eredmények (2011–2018. augusztus). A vizsgált időszakban összesen 927 beteghez tartozó 1070 szérum és/vagy CSF mintában vizsgáltuk az autoantitestek jelenlétét. A 69 AE pozitív betegben detektált autoantitestek megoszlását ábrázolja a kördiagram (NMDAR>LGI1>GABA$_B$R>Caspr2). Vizsgálatunk során AMPAR1 és AMPR2 elleni autoantitesteket egyik mintában sem találtunk



1. táblázat. A neuromyelitis optica (NMO), az autoimmun encephalitisek (AE) és a paraneoplasiás neurológiai szindrómák (PNS) klinikai jellemzői, a jellegzetes autoantitestek és ezek detektálására szolgáló labordiagnosztikai módszerek. Laboratóriumunkban NMO és AE esetén az autoantitestek detektálását sejt alapú indirekt immunfluoreszcens BIOCHIP esszével végezzük (NMO: szérum, AE: szérum és liquor), míg PNS esetén az onkoneurális ellenanyagok kimutatása rekombináns fehérje antigénnel ellátott immunoblot esszével (szérum) történik.

Antitest	Érintett betegcsoportok	Szindróma	Kapcsolódó daganat
NEUROMYELITIS OPTICA (NMO) ÉS NMO SPEKTRUM BETEGSÉGEK			
Labordiagnosztikai módszer: sejtes BIOCHIP (transzfektált sejtvonalak)			
anti-AQP4 anti-MOG	Leggyakrabban fiatal nők (medián: 30–40 év)	Optikus neuritis (látásélesség-csökkenés, látótérkiesések, súlyos látászavar, mely rövid idő alatt akár vakságig progrediálhat); myelitis (paraplegia / tetraplegia, érzészavarok, vizeletretenció, nyúltvelőbe terjedés esetén légzésbénulás)	Ritkán: thymoma, egyéb
AUTOIMMUN ENCEPHALITISEK (AE)			
Labordiagnosztikai módszer: sejtes BIOCHIP (transzfektált sejtvonalak)			
anti-NMDAR	Leggyakrabban fiatal nők (medián: 21 év), de gyermekekben és fiúkban is egyre gyakoribb	Infekciós-influenzaszerű prodroma Pszichiátriai tünetek (agitáció, hallucinációk, delúziók, katatónia, pszichózis) Késői manifesztációk (kognitív és beszédzavarok, orofaciális dyskinesia, epilepsziás rosszullétek, autonóm zavarok, kóma)	10–50%, ovárium/ hereteratoma
anti-LGI1	Idős férfiak (medián: 64 év)	Faciobrachialis dystoniás görcsök, limbikus encephalitis, hyponatraemia, alvászavarok, memória- és kognitív zavarok	<5%, thymoma
anti-GABA$_B$R	Férfi dominancia (medián: 61 év)	Limbikus encephalitis, epilepsziás rohamok Ritkán: cerebelláris ataxia, opsoclonus-myoclonus	50%, SCLC
anti-Caspr2	Idős férfiak (medián: 66 év)	Neuromyotonia, Morvan-szindróma, limbikus encephalitis, inszomnia, neuropátiás fájdalom	<5%, thymoma
anti-AMPAR1 és 2	Női dominancia (medián: 56 év)	Limbikus encephalitis, epilepsziás rohamok Ritkán: pszichiátriai tünetek	10–50%, SCLC, thymoma vagy emlő
PARANEOPLASZTIKUS NEUROLÓGIAI SZINDRÓMÁK (PNS)			
Labordiagnosztikai módszer: immunoblot rekombináns tisztított antigénekkel			
anti-Ma2	Többségük felnőtt (40–70 év) Férfiak és nők egyenlő arányban érintettek	Limbikus, agytörzsi, hypothalamicus encephalitis Ritkán: cerebelláris degeneráció	Heretumor (40 év alatti férfiak), egyéb szolid tumorok
anti-Yo		Cerebelláris degeneráció	Ovárium, emlő
anti-CV2		Encephalomyelitis, cerebelláris degeneráció, chorea, perifériás neuropátia, uveitis	SCLC, thymoma, egyéb
anti-Hu		Encephalomyelitis (kortikális, limbikus, agytörzsi encephalitis), cerebelláris degeneráció, myelitis, szenzoros neuropátia, autonóm diszfunkció	SCLC, egyéb
anti-amphiphysin		Stiff-person szindróma, encephalomyelitis, limbikus encephalitis, myelopathia	SCLC, emlő
anti-Ri		Cerebelláris degeneráció, agytörzsi encephalitis, opsoclonus-myoclonus	Emlő, ovarium, SCLC

LABORDIAGNOSZTIKAI MÓDSZEREK

A molekuláris biológiai módszerek fejlődése tette lehetővé a laboratóriumunkban alkalmazott diagnosztikai eljárások kifejlesztését.

Folytatás a 15. oldalon

Folytatás a 14. oldalról

A célantigének tisztításával lehetőség nyílt a rekombináns fehérjéket tartalmazó immunoblot kifejlesztésére (PNS), valamint az érintett struktúrák génjeinek pontos megismerése tette lehetővé a különböző antigének génjeivel transzfektált sejtalapú esszék (NMO/NMOSD, AE) létrejöttét.

Laboratóriumunkban az anti-AQP4 és anti-MOG autoantitestek detektálását sejtalapú indirekt immunfluoreszcens BIOCHIP esszével végezzük. Az 5 minta vizsgálatára alkalmas BIOCHIP lemez az AQP4 és MOG molekulákat kódoló DNS-sel transzfektált és a vizsgálni kívánt antigéneket tömegesen expresszáló HEK293 sejteket tartalmazza (9). Kezdetben kizárólag az anti-AQP4 autoantitestek kimutatására volt lehetőség, azonban az elmúlt évben bevezették az anti-AQP4 és anti-MOG autoantitestek egyidejű detektálására alkalmas esszét, mely még pontosabb differenciáldiagnosztikát tesz lehetővé NMO/NMOSD gyanús betegek esetén. A vizsgálatot minden esetben szérumból végezzük (10-szeres hígításban), melyet a BIOCHIP lemezzel inkubálunk, majd anti-humán IgG-FITC-cel jelölt másodlagos ellenanyaggal mutatjuk ki a transzfektált sejtekhez kötődött autoantitest jelenlétét fluoreszcens mikroszkóp segítségével.

Laboratóriumunkban az AE-specifikus ellenanyagok kimutatását szintén sejtalapú indirekt immunfluoreszcens BIOCHIP esszével végezzük. Itt a BIOCHIP lemezeken az AE-re jellemző 6 antigénnel (NMDAR, LGI1, GABABR, Caspr2, AMPAR1, AMPAR2) transzfektált HEK293 sejtek találhatók (9). A teszt kivitelezésének lépései megegyeznek az NMO-nál részletezett leírással azzal az eltéréssel, hogy AE esetén a szérum és a cerebrospinális folyadék (CSF) párhuzamos vizsgálata ajánlott, valamint liquor esetén nem alkalmazunk hígítást.

Laboratóriumunkban az onkoneurális ellenanyagok kimutatását immunoblot esszével végezzük, mely 6 (Ma2, Yo, Hu, CV2, amphiphysin, Ri), illetve 12 (az előzőleg említett 6 típuson kívül Tr, GAD65, Zic4, titin, SOX1, recoverin) rekombináns fehérjeantigént tartalmaz meghatározott lokalizációban. A vizsgálatot minden esetben szérumból végezzük (10-szeres hígításban), melyet az antigénekkel ellátott membráncsíkokkal inkubálunk, majd anti-humán IgG-HRPO jelölt másodlagos ellenanyag hozzáadását követően a színreakció jelenlétét, illetve erősségét EUROLine-Scan program segítségével értékeljük.

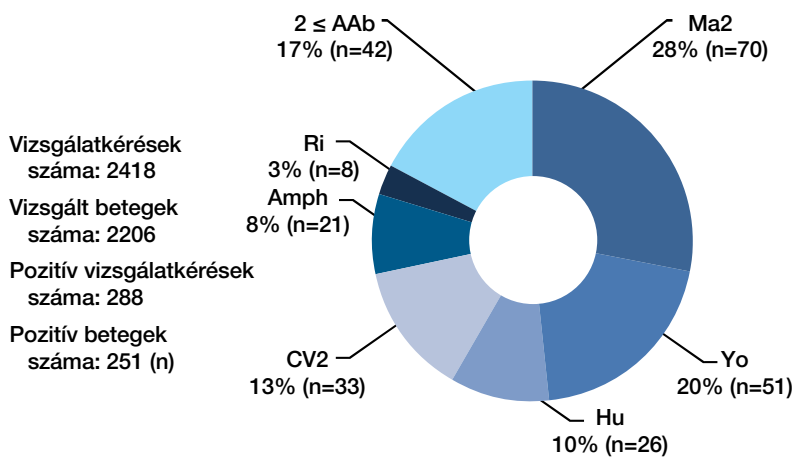
VIZSGÁLATI EREDMÉNYEINK, KÖVETKEZTETÉSEK

Laboratóriumunkba a 2011–2018. szeptember közötti időszakban 1997 NMO/NMOSD irányú vizsgálatkérés érkezett az anti-AQP4 autoantitestek detektálása céljából. A vizsgált szérumminták 7,4%-ában (153 pozitív eredménnyel zárult vizsgálatkérés) találtunk anti-AQP4 ellenanyagot.

Az AE és PNS irányú vizsgálatkérések száma (Magyarországon számos neurológiai osztályról kaptunk az egész országot lefedő betegmintákat) a tesztek bevezetése óta (AE: 2011, PNS: 2010) folyamatosan emelkedő tendenciát mutat. A vizsgált időszakban összesen 1070 AE irányú vizsgálatkérés érkezett 927 beteghez tartozóan (2011–2018. augusztus), valamint 2418 PNS tesztet végeztünk el 2206 beteghez tartozóan (2010–2018. augusztus). Az AE irányában történő vizsgálatok során 69 személynél detektáltunk autoantitestet a szérum- és/vagy CSF-mintákban, az alábbi gyakorisági sorrendben: NMDAR (78%), LGI1 (13%), GABABR (9%) és Caspr2 (3%). A minták tesztelése során AMPAR1 és AMPR2 elleni autoantitesteket egyik esetben sem találtunk *(1.A ábra)*. A PNS lehetséges fennállásának irányában végzett vizsgálatok során 251 személynél találtunk autoantitestet a szérummintákban, az alábbi gyakorisági sorrendben: Ma2 (28%), Yo (20%), CV2 (13%), Hu (10%), amphiphysin (8%) és Ri (3%). A pozitív betegek 17%-ában egyidejűleg nemcsak 1, hanem 2 vagy 3 fajta autoantitestre találtunk pozitivitást (1.B ábra).

A vizsgálatunk során kapott adatokat egybehangzónak találtuk a szakirodalommal az AE és a PNS prevalenciája és a betegségek nem és életkor szerinti megoszlása kapcsán. Az NMDAR encephalitis a leggyakoribb típus, mely általában fiatal nőket érint, ezt követi a középkorú férfiakban gyakori LGI1 encephalitis és végül a GABABR encephalitis, mely általában idősebb férfiakban fordul elő. A PNS betegek esetén nem volt jelentős különbség a nemek közötti megoszlás tekintetében; a nők és a férfiak körülbelül egyenlő arányban érintettek, és a kórkép általában az idősebb korosztályban jelentkezett. AE esetén vizsgálati eredményeink igazolták, hogy NMDAR encephalitis kapcsán az autoantitestek jobban detektálhatóak a liquorban a szérumhoz képest (a liquor általában erős pozitív, a szérum gyenge pozitív, de akár negatív is lehet), míg LGI1 encephalitis kapcsán előfordulhat, hogy az ellenanyagok kizárólag a szérumban vagy a liquorban mutathatók ki. Így AE gyanúja esetén a szérum és a CSF egyidejű vizsgálata javasolt. Az AE-vel ellentétben NMO vagy PNS esetén elegendő kizárólag a szérum vizsgálata az autoantitestek detektálásához.

A részletezett labordiagnosztikai eljárások ismerete nemcsak a laboratóriumi szakemberek számára fontos, de a klinikusok (neurológusok, pszichiáterek, onkológusok) számára is elengedhetetlen, mivel a neuromyelitis optica, az autoimmun encephalitisek és a paraneoplasiás neurológiai szindrómák pontos klinikai diagnosztikájának elengedhetetlen részét képezik. A betegségekre jellemző autoantitestek detektálása leghatékonyabban a laboratóriumi szakemberek és a klinikusok megfelelő együttműködésével valósulhat meg. Kiemelt jelentőséggel bír a megfelelő mintatípus megválasztása (AE: szérum és liquor, NMO és PNS: szérum), mely a laboratóriumi tapasztalatokon alapul.



1.B ábra: A paraneoplasiás neurológiai szindróma irányában vizsgált betegek mintáinak feldolgozása során kapott eredmények (2010–2018. augusztus). A vizsgált időszakban összesen 2206 beteghez tartozó 2418 vizsgálatkérés érkezett. 251 betegnél detektáltunk onkoneurális autoantitestet a szérumban, ezen ellenanyagok megoszlását ábrázolja a kördiagram (Ma2>Yo>CV2>Hu>amphiphysin>Ri). 42 pozitív betegnél egyidejűleg 2 vagy 3 fajta autoantitestet (AAb) is detektáltunk

KÖSZÖNETNYILVÁNÍTÁS:
A kézirat a GINOP-232-15-2016-00050 és az EFOP 361-16-2016-00004 pályázat támogatásával készült.

BERKI TÍMEA, HAYDEN ZSÓFIA, BÖRÖCZ KATALIN, CSIZMADIA ZSUZSANNA, KELLERMAYER ZOLTÁN, BALOGH PÉTER
Pécsi Tudományegyetem, Általános Orvostudományi Kar, Klinikai Központ, Immunológiai és Biotechnológiai Intézet, Pécs

IRODALOM

1. Patterson SL, Goglin SE. Neuromyelitis Optica. Rheum Dis Clin North Am. 2017;43(4):579–91.
2. Pröbstel AK, Rudolf G, Dornmair K, Collongues N, Chanson JB, Sanderson NS, et al. Anti-MOG antibodies are present in a subgroup of patients with a neuromyelitis optica phenotype. J Neuroinflammation. 2015;12:46.
3. Zuliani L, Graus F, Giometto B, Bien C, Vincent A. Central nervous system neuronal surface antibody associated syndromes: review and guidelines for recognition. J Neurol Neurosurg Psychiatry. 2012;83(6):638–45.
4. Dalmau J, Graus F. Antibody-Mediated Encephalitis. N Engl J Med. 2018;378(9):840–51.
5. Hughes EG, Peng X, Gleichman AJ, Lai M, Zhou L, Tsou R, et al. Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. J Neurosci. 2010;30(17):5866–75.
6. Ohkawa T, Fukata Y, Yamasaki M, Miyazaki T, Yokoi N, Takashima H, et al. Autoantibodies to epilepsy-related LGI1 in limbic encephalitis neutralize LGI1-ADAM22 interaction and reduce synaptic AMPA receptors. J Neurosci. 2013;33(46):18161–74.
7. Rosenfeld MR, Dalmau J. Paraneoplastic Neurologic Syndromes. Neurol Clin. 2018;36(3):675–85.
8. Höftberger R, Rosenfeld MR, Dalmau J. Update on neurological paraneoplastic syndromes. Curr Opin Oncol. 2015;27(6):489–95.
9. Naides SJ. The role of the laboratory in the expanding field of neuroimmunology: Autoantibodies to neural targets. J Immunol Methods. 2018;463:1–20.

A paraneoplásiás neurológiai szindrómák laboratóriumi diagnosztikája és immunológiai vonatkozásai

HAYDEN ZSÓFIA, BÖRÖCZ KATALIN, CSIZMADIA ZSUZSANNA, KELLERMAYER ZOLTÁN, BALOGH PÉTER, BERKI TÍMEA

Pécsi Tudományegyetem, Általános Orvostudományi Kar, Klinikai Központ, Immunológiai és Biotechnológiai Intézet, Pécs

Levelezési cím:
Prof. Dr. Berki Tímea, Pécsi Tudományegyetem, Általános Orvostudományi Kar, Klinikai Központ, Immunológiai és Biotechnológiai Intézet, 7624 Pécs, Szigeti út 12. E-mail: berki.timea@pte.hu, telefon: 06 72/536-288, 36291 mellék

Közlésre érkezett:
2019. május 31.
Elfogadva:
2019. július 18.

A paraneoplásiás neurológiai szindrómák (PNS) és az autoimmun enkefalitiszek (AE) ritka idegrendszeri kórképek, melyek hasonló tünettannal jelentkeznek az érintett betegekben, azonban prognózisuk és kezelésük eltér. Jelenlegi retrospektív statisztikai elemzésünk során 2362 PNS- és 1034 AE-gyanús beteg szérum- és likvormintái laboratóriumi teszt eredményeinek értékelését végeztük el. Az autoantitestek kimutatása céljából PNS esetén line-immunoblot assay-t, AE esetén sejtalapú indirekt immunfluoreszcens assay-t alkalmaztunk. Analízisünk során a PNS-gyanús betegek 8%-ában találtunk autoantitestet a következő megoszlással: anti-Yo > anti-Hu > anti-Ma2 > anti-CV2 > anti-titin > anti-Zic4 > anti-amfifizin > anti-Ri > anti-GAD65 > anti-Sox1 > anti-recoverin, melyek előfordulása idősebb nőkben volt gyakoribb. Az AE-gyanús betegek 5,8%-ában találtunk autoantitestet: anti-NMDAR (fiatal nőkben) > anti-LGI1 (középkorú férfiakban) > anti-$GABA_BR$ (idősebb férfiakban) > anti-Caspr2 (felnőtt férfiakban). Eredményeink az autoantitestek gyakoriságát, nem és életkor szerinti megoszlását tekintve a szakirodalmi adatokkal nagyfokú hasonlóságot mutattak. A PNS és AE diagnózisának felállítását segíti az autoantitestek kimutatása, amely a modern labordiagnosztikai módszerek bevezetésével egyre több beteg számára lehetővé teszi a kezelés korai bevezetését. Magy Onkol 63:261-267, 2019

Kulcsszavak: paraneoplásiás neurológiai szindrómák, autoimmun enkefalitisz, autoantitest, laboratóriumi diagnosztika

Paraneoplastic neurologic syndromes (PNS) and autoimmune encephalitis (AE) are rare neurological disorders, which have similar symptoms, but vary in outcome and treatment strategy. In our retrospective statistical study we evaluated the autoantibody test results of serum and CSF from 2362 patients with suspected PNS and 1034 patients with suspected AE. For autoantibody testing, immunoblot assay (PNS) and cell-based indirect immunofluorescence assay (AE) were used. Autoantibodies were present in 8% of patients with suspected PNS: anti-Yo > anti-Hu > anti-Ma2 > anti-CV2 > anti-titin > anti-Zic4 > anti-amphiphysin > anti-Ri > anti-GAD65 > anti-Sox1 > anti-recoverin. Mostly elderly women were affected. Autoantibodies were present in 5.8% of patients with suspected AE: anti-NMDAR (young women) > anti-LGI1 (middle-aged men) > anti-$GABA_BR$ (elderly men) > anti-Caspr2 (adult men). Our results correspond to the data described in the literature. The number of patients with suspected PNS and AE shows an increasing tendency, where the autoantibody testing with modern laboratory diagnostic methods helps in the early introduction of the appropriate therapy.

Hayden Z, Böröcz K, Csizmadia Z, Kellermayer Z, Balogh P, Berki T. Paraneoplastic neurologic syndromes: laboratory diagnostics and immunological aspects. Magy Onkol 63:261-267, 2019

Keywords: *paraneoplastic neurologic syndromes, autoimmune encephalitis, autoantibody, laboratory diagnostics*

BEVEZETÉS

A paraneoplásiás neurológiai szindrómák (PNS) ritka kórképek, melyek daganatos betegséghez társulnak. Előfordulási gyakoriságuk a daganatos betegekben 0,5–1%-ra tehető. Kialakulásukért nem a tumor vagy a metasztázis direkt hatása vagy a daganat ellen irányuló terápia felelős, illetve egyéb metabolikus és vaszkuláris tényezők, valamint egy fennálló infekció kóroki szerepe is egyértelműen kizárható. A háttérben egy autoimmun folyamat áll, melynek alapja a molekuláris mimikri. Az intracelluláris antigének (Ma2, Yo, CV2, Hu, amfifizin, Ri, Tr, GAD65, Zic4, titin, SOX1, recoverin), melyek ellen az autoantitestek képződnek, fiziológiás körülmények között a neuronális szövetekben is megjelennek. Így a tumor ellen generált celluláris és humorális immunválasz a strukturálisan azonos, neuronális antigénekkel is keresztreagál, mely neurológiai tünetek kialakulásához vezet. Az immunválasz során a $CD8^+$ T-sejtek a tumor által expresszált antigénekhez kapcsolódva a tumorsejt líziséhez vezetnek. A felszabaduló antigéneket az antigénprezentáló sejtek a regionális nyirokcsomókba szállítják, ahol bemutatják azokat a $CD4^+$ T-sejteknek. Az antigénnel való találkozás hatására aktiválódott $CD4^+$ T-sejtek egyrészt keresztreagálnak a központi idegrendszerben is megjelenő neuronális antigénekkel és gyulladáshoz vezetnek, másrészt aktiválják a B-sejteket, melyek a tumorantigének ellen antitesteket termelnek. Ezen ellenanyagok komplementaktiváció vagy kompetitív gátlás által a neuronális struktúrákat is károsítják. Ezzel egyidejűleg a $CD8^+$ T-sejtek irreverzibilis neuronpusztulást idéznek elő. A képződött ellenanyagok patológiás szerepe nem teljesen bizonyított, főleg a $CD8^+$ T-sejtek játszanak szerepet a betegség kialakulásában (1). A klasszikus paraneoplásiás neurológiai szindrómákkal szemben, melyekben a termelt antitestek intracelluláris antigének ellen irányulnak, autoimmun enkefalitisz (AE) esetén az ellenanyagok sejtfelszíni és szinaptikus proteinek ellen képződnek.

A képződött antitestek felosztása terápiás jelentőséggel bír, hiszen AE esetén a klasszikus paraneoplásiás neurológiai szindrómákkal szemben az immunterápia hatékonynak bizonyul az esetek döntő többségében, illetve az érintettek prognózisa is kedvezőbb (2). A szindróma időbeni felismerése fontos, az immunterápia megkezdéséhez is elengedhetetlen, melynek hiánya súlyos maradványtünetekhez vezethet az érintett betegekben. Az autoantitestek mérése nemcsak diagnosztikus jelentőségű, hanem alkalmas a betegség lefolyásának, illetve a terápia hatékonyságának nyomon követésére is. PNS esetén az autoantitestek biomarkerként egy okkult tumort jelezhetnek, ezért kimutatásuk rendkívül fontos, hiszen a neurológiai tünetek az esetek kétharmadában megelőzik a daganat diagnózisát. A PNS diagnózisa három fő pilléren nyugszik: 1. a neurológiai kórkép felismerése, 2. a szindrómára jellegzetes ellenanyag vizsgálata, 3. tumorkutatás, negativitás esetén akár négy évig ismételve (3, 4). A neurológiai tünetek a centrális és perifériás idegrendszer bármely részét érinthetik, beleértve a retinát és a vázizmokat. Az egyes ellenanyagokhoz társuló szindrómát, illetve a leggyakrabban asszociálódó tumortípusokat az *1. táblázatban* foglaltuk össze.

ANYAG ÉS MÓDSZER

Laboratóriumunkban a 2010 és 2018 közötti időszakban 2362 PNS- és 1034 AE-gyanús beteg szérum- és likvorvizsgálati eredményeinek értékelését végeztük el egy retrospektív statisztikai analízis keretében. A vizsgálatot a Pécsi Tudományegyetem Regionális Kutatásetikai Bizottságának jóváhagyásával végeztük (RIKEB 6966/2017).

Onkoneurális antitestek mérése line-immunoblottal

Az intracelluláris antigének ellen képződő ún. onkoneurális ellenanyagok vizsgálatát line-immunoblot assay-vel végezzük. Laboratóriumunkban kétfajta kitet alkalmazunk: 2010-ben a klasszikus paraneoplásiás neurológiai szindrómára jellemző 6-féle különböző tisztított rekombináns fehérjeantigént (Hu, Yo, Ri, CV2, Ma2, amfifizin) tartalmazó tesztet vezettük be, majd 2016-ban egy 12 antigént tartalmazó line-blot is alkalmazásra került laboratóriumunkban, mely az addigi 6 autoantitesten kívül további 6 jellegzetes autoantitest (Tr, GAD65, Zic4, titin, SOX1, recoverin) kimutatását tette lehetővé. A teszt segítségével egy mintában egyidejűleg 6, illetve 12 fajta különböző autoantitest detektálására van lehetőség. A vizsgálat kivitelezése a gyártó által mellékelt protokoll alapján történik, szérummintából. A blot csíkokat 1,5 ml mennyiségű hígított szérummintával (1:101 hígítás) inkubáljuk 30 percig szobahőmérsékleten körkörös rázással. Ezt követően 3 mosási lépést végzünk, majd 1,5 ml hígított anti-humán IgG-HRPO másodlagos ellenanyaggal inkubáljuk a blot csíkokat 30 percig szobahőmérsékleten. 3 mosási lépés után 1,5 ml szubsztrátoldat hozzáadása történik 10 percig, majd egy desztillált vizes mosás után a tesztcsíkokat az értékeléshez használt lapra rögzítjük és hagyjuk megszáradni, majd a kapott eredményt EUROLineScan program segítségével értékeljük. A vizsgálat során pozitív és negatív kontrollt használunk. Az értékeléshez használt szkennelő 2017-ben került bevezetésre, és a színreakció jelenléte, illetve erőssége alapján képes elkülöníteni a pozitív és negatív vizsgálati eredményeket a kontrollok segítségével. Az eredmények beolvasása során a program minden színreakcióhoz rendel egy számértéket a detektált reakció erőssége alapján egy 0-tól 50-ig terjedő skálán. A kapott számértékeket a kitben mellékelt értéktartományok alapján 4 csoportba soroljuk a leletkiadás során: 0–5 érték negatív, 6–10 érték kétes, 11–25 gyengén pozitív (+), 26–50 érték pozitív (++) és >50 érték erősen pozitív (+++) eredmény. Jelen munkánkban a 10 alatti értékeket negatívnak, a 10 fölötti értékeket pozitívnak értékeltük.

AE-re jellemző autoantitestek mérése sejtalapú BIOCHIP-pel

A sejtfelszíni, illetve szinaptikus proteinek ellen képződő ellenanyagok detektálását sejtalapú indirekt immunfluoreszcens BIOCHIP assay-vel végezzük. A BIOCHIP-en a 6-féle

1. TÁBLÁZAT. A paraneoplázias neurológiai szindrómák jellemzői

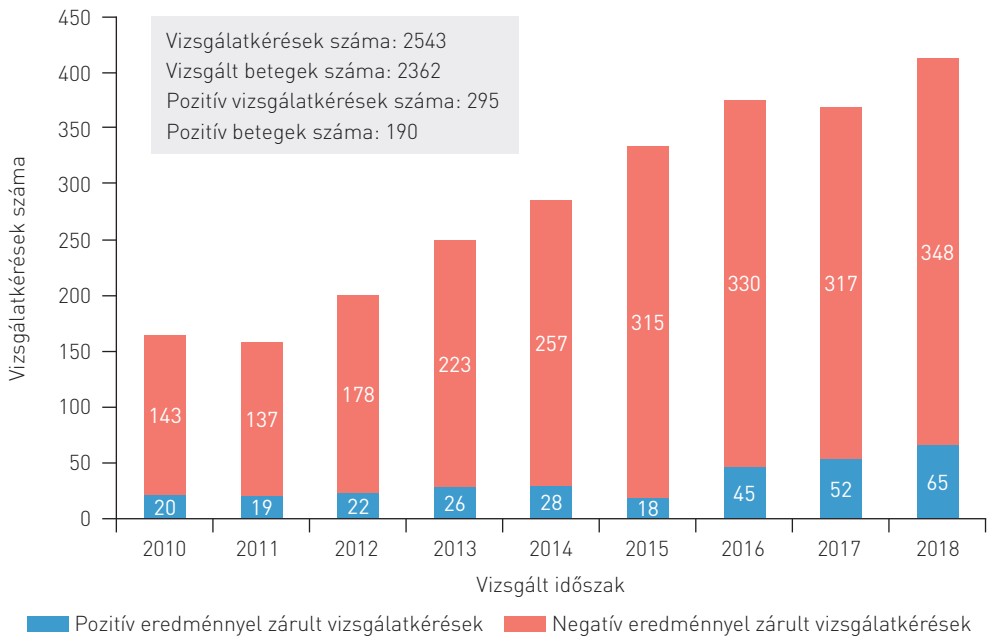
Antigén	Szindróma	Kapcsolódó daganat
Intracelluláris antigének		
Hu	enkefalomielitisz (kortikális, limbikus, agytörzsi enkefalitisz), cerebelláris degeneráció, mielitisz, szenzoros neuropátia, autonóm diszfunkció	SCLC, egyéb
Yo	cerebelláris degeneráció	petefészek, emlő
Ri	cerebelláris degeneráció, agytörzsi enkefalitisz, opszoklónusz-mioklónusz	emlő, petefészek, SCLC
Tr	cerebelláris degeneráció	Hodgkin-limfóma
CV2/CRMP5	enkefalomielitisz, cerebelláris degeneráció, chorea, perifériás neuropátia, uveitisz	SCLC, timóma, egyéb
Ma2	limbikus, agytörzsi, hipotalamikus enkefalitisz, ritkán: cerebelláris degeneráció	heretumor (40 év alatti férfiak), egyéb szolid tumorok
Amfifizin	stiff-person-szindróma, enkefalomielitisz, limbikus enkefalitisz, mielopátia	SCLC, emlő
Recoverin	retinopátia	SCLC
Zic4	cerebelláris degeneráció	SCLC
Titin	myasthenia gravis	timóma
Sox1	Lambert-Eaton miaszténiás szindróma, neuropátia	SCLC
GAD65	limbikus enkefalitisz, stiff-person-szindróma, cerebelláris ataxia, opszoklónusz-mioklónusz	SCLC, neuroendokrin, timóma, emlő, non-Hodgkin-limfóma
Sejtmembránantigének		
NMDAR	enkefalitisz	petefészek/here teratóma
$GABA_BR$	limbikus enkefalitisz	SCLC
AMPAR1,2	limbikus enkefalitisz, epilepsziás rohamok, ritkán: pszichiátriai tünetek	SCLC, timóma, emlő
Caspr2	neuromiotónia, Morvan-szindróma, limbikus enkefalitisz, inszomnia, neuropátiás fájdalom	timóma
Extracelluláris antigének		
LGI1	limbikus enkefalitisz, faciobrahiális disztóniás rohamok	timóma

A táblázat a paraneoplázias neurológiai szindrómákban leggyakrabban előforduló autoantitesteket tartalmazza. Az egyes ellenanyagokhoz jellegzetes neurológiai tünetek és meghatározott típusú tumorok társulnak. SCLC: small cell lung cancer (kissejtes tüdőrák)

receptorfehérje (NMDAR, LGI1, Caspr2, $GABA_BR$, AMPAR1, AMPAR2) génjeivel transzfektált, illetve azokat expresszáló HEK293 sejtek találhatók. A BIOCHIP egy mozaikja 6 különböző neuronális antigént expresszáló sejtvonalat tartalmaz és rajta egyidejűleg 6 különböző autoantitest kimutatása lehetséges. Az assay kivitelezése a gyártó által mellékelt protokoll alapján történik. A 30 µl mennyiségű minta (szérum esetén 1:10 hígításban, likvor esetén hígítatlanul) felvitelét követően 30 perces inkubáció történik szobahőmérsékleten. Ezt követően 2 mosási lépést végzünk PBS-Tween 20 pufferrel 5 percig, melyet 25 µl anti-humán IgG-FITC másodlagos ellenanyaggal történő inkubálás követ 30 percig szobahőmérsékleten. Majd 2 mosási lépést követően a lemezt glicerinnel fedjük le és a kapott eredményt fluoreszcens mikroszkóp (Olympus Bx61) alatt értékeljük Zeiss Axiocam 305 kamera segítségével. A BIOCHIP assay-k értékelése minden esetben legalább két független laboratóriumi szakember által történik. A vizsgálat során pozitív és negatív kontrollt használunk, és a mikroszkóp alatt detektált reakció erőssége alapján a kapott eredményt az erősen pozitív, pozitív, gyengén pozitív, kétes vagy negatív csoportba soroljuk.

EREDMÉNYEK

Az onkoneurális ellenanyagok kimutatására alkalmas teszt bevezetése óta (2010) a vizsgálatkérések száma folyamatosan emelkedő tendenciát mutat. A 2010 és 2018 közötti vizsgált időszakban laboratóriumunkba összesen 2543 PNS irányú vizsgálatkérés érkezett 2362 beteghez tartozóan. Vizsgálatunk során 190 beteghez tartozó 235 szérummintát találtunk pozitívnak, tehát a PNS-gyanús betegek 8%-ában



1. ÁBRA. A paraneopláziás neurológiai szindróma irányú vizsgálatkérések alakulása a 2010–2018 közötti időszakban. Az oszlopdiagramok a pozitív és negatív eredménnyel zárult vizsgálatokat ábrázolják éves felosztásban. Emellett feltüntettük az összes és a pozitív eredménnyel zárult vizsgálatkérések és a vizsgált betegek számát is a teljes vizsgált időszakban

detektáltunk autoantitest-pozitivitást. A vizsgált időszakban változó gyakorisággal találtunk pozitivitást: 12,3% (2010), 12,2% (2011), 11% (2012), 10,4% (2013), 9,8% (2014), 5,4% (2015), 12% (2016), 14,1% (2017), 15,7% (2018). A pozitív vizsgálatok aránya 2018-ban volt a legmagasabb (15,7%), míg 2015-ben volt a legalacsonyabb (5,4%) *(1. ábra).* 2016-tól a vizsgálatkérések, valamint a pozitív esetek száma egyaránt emelkedést mutat a korábbi évekhez képest. A magasabb pozitivitás hátterében áll, hogy a 6-os line-blot mellett 2016-ban került bevezetésre laboratóriumunkban a 12 antigén egyidejű vizsgálatára alkalmas line-blot, mellyel az onkoneurális ellenanyagok szélesebb körének tesztelése vált lehetővé. A 190 pozitív betegben az alábbi gyakorisági sorrendben mértünk autoantitestet: anti-Yo (25%), anti-Hu (20%), anti-Ma2 (19%), anti-CV2 (12%), anti-titin (10%), anti-Zic4 (9%), anti-amfifizin (7%), anti-Ri (3%), anti-GAD65 (3%), anti-Sox1 (3%) és anti-recoverin (1%). Vizsgálatunk során nem találtunk pozitivitást az anti-Tr autoantitestre *(2. táblázat).* Húsz beteg esetén két különböző autoantitestre találtunk pozitivitást egyidejűleg. Vizsgálatunk során 141 beteghez tartozó 151 vizsgálatkérés kétes eredménnyel zárult.

Statisztikai analízisünk során vizsgáltuk a PNS-specifikus ellenanyagok nem és életkor szerinti megoszlását. A 190 PNS-specifikus ellenanyagra pozitív beteg medián életkora 62 év (16–88 év), a pozitív betegek közül 120 nő és 70 férfi. A PNS-betegek nem és életkor szerinti megoszlására és a különböző autoantitestek incidenciájára vonatkozó adatokat a *2. táblázat* foglalja össze.

A sejtfelszíni/szinaptikus proteinek ellen képződő ellenanyagok kimutatására alkalmas teszt bevezetése óta (2012) az autoimmun enkefalitisz irányú vizsgálatkérések száma szintén folyamatosan emelkedő tendenciát mutat. A 2012 és 2018 közötti vizsgált időszakban laboratóriumunkba összesen 1247 AE irányú vizsgálatkérés érkezett 1034 beteghez tartozóan. Vizsgálatunk során 60 beteghez tartozó 98 szérum- és/vagy likvormintát találtunk pozitívnak, tehát az AE-gyanús betegek 5,8%-ában detektáltunk autoantitest-pozitivitást. A vizsgált időszakban változó gyakorisággal láttunk pozitivitást, a legtöbb pozitív eredményű vizsgálatkérés a teszt bevezetését követő 4 éven belül érkezett laboratóriumunkba [22,4% (2012), 18,7% (2013), 6,1% (2014), 13,4% (2015)]. Bár az elmúlt 3 évben az AE irányú vizsgálatkérések száma emelkedő tendenciát mutat, a pozitivitás aránya alacsonyabb volt a korábbi évekhez képest [4,3% (2016), 3% (2017), 4,4% (2018)]. A legtöbb pozitív eredményű vizsgálatkérés 2012-ben érkezett laboratóriumunkba (22,4%), míg 2017-ben volt a legalacsonyabb a pozitív teszteredmények aránya *(2. ábra).* A 60 pozitív betegben az alábbi gyakorisági sorrendben fordultak elő autoantitestek: anti-NMDAR (70%), anti-LGI1 (15%), anti-$GABA_BR$ (12%) és anti-Caspr2

2. TÁBLÁZAT. A laboratóriumunkban 2010–2018 között talált, onkoneurális autoantitestre pozitív betegek jellemzői

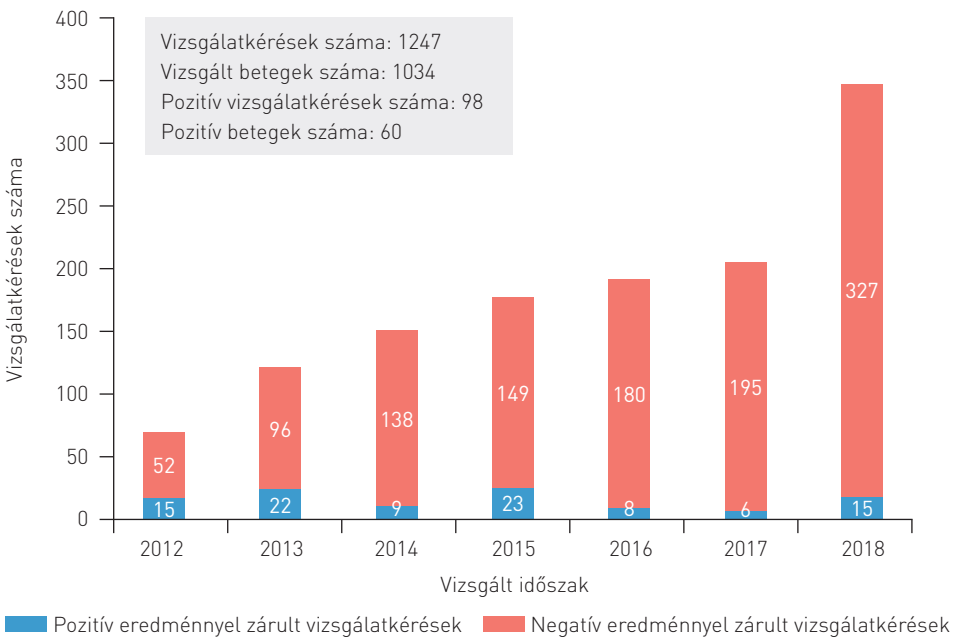
Autoantitest	Pozitív betegek száma	Férfi:nő	Életkor (év) medián (tartomány)	Incidencia/100 000 lakos
A 2010–2018-as időszakban vizsgált klasszikus onkoneurális antigének (2362 beteg)				
Yo	47	0,5:1	63 (16–86)	0,47
Hu	38	0,7:1	64 (21–84)	0,38
Ma2	36	0,5:1	63 (19–88)	0,36
CV2	23	0,8:1	62 (23–73)	0,23
Amfifizin	14	1:1	62 (35–74)	0,14
Ri	5	1:0,7	60 (49–60)	0,05
A 2016–2018-as időszakban vizsgált újabb antigének (358 beteg)				
Titin	18	0,6:1	66 (38–83)	0,18
Zic4	17	0,5:1	62 (32–79)	0,17
GAD65	5	0,7:1	61 (31–77)	0,05
Sox1	5	0,7:1	53 (46–61)	0,05
Recoverin	2	1:1	62 (58–66)	0,02
Tr	0	–	–	–

A táblázatban az egyes autoantitestekre pozitív betegek száma, nem és életkor szerinti megoszlása és az adott időszakra vonatkozó incidenciája olvasható. Húsz beteg esetén egyidejűleg két fajta autoantitestre találtunk pozitivitást

(7%). Vizsgálatunk során nem találtunk pozitivitást az anti-AMPAR1 és anti-AMPAR2 autoantitestekre *(3. táblázat).* Két beteg egyidejűleg két különböző autoantitestre is pozitívnak bizonyult: az egyik betegben LGI1 és Caspr2 elleni autoantitesteket, míg a másik beteg esetén anti-$GABA_BR$ és anti-Caspr2 ellenanyagokat detektáltunk. Vizsgálatunk során 12 beteg szérummintájának vizsgálata kétes eredménnyel zárult (11 NMDAR és 1 $GABA_BR$). E betegcsoportból 5 betegnél a szérumminta vizsgálata mellett likvormintából is elvégeztük az autoantitest-mérést, mely minden esetben negatív eredménnyel zárult. A kétes eredményű betegek közül 3 esetben újabb szérummintát küldtek laboratóriumunkba, azonban az ismételt vizsgálat során mindegyik minta kapcsán negativitást találtunk.

A 60 pozitív beteg közül 34 esetben vagy csak szérum- (28 eset), vagy csak likvormintákból (6 esetben) történt vizsgálat, míg 26 beteg esetén (17 anti-NMDAR-, 3 anti-LGI1-, 4 anti-$GABA_BR$-, 1 anti-Caspr2-, 1 anti-LGI1- és anti-Caspr2-pozitív beteg) mindkét minta egyidejű vizsgálata történt. A 17 anti-NMDAR-pozitív beteg vizsgálata során 3 esetben kizárólag a szérumban, 6 esetben kizárólag a likvorban, míg 8 esetben mindkét mintatípusban mértük az autoantitestek jelenlétét. 3 beteg esetében a likvorban erősebb pozitivitást találtunk a szérumhoz képest. A 3 anti-LGI1-pozitív beteg kapcsán kizárólag a szérum vizsgálata zárult pozitív eredménnyel. A 4 anti-$GABA_BR$ autoantitestre pozitív beteg vizsgálata során mindkét mintatípusban kimutattuk az autoantitestet, egy alkalommal erősebb reakciót mutatott a szérum vizsgálata a likvorhoz képest. Az anti-Caspr2 ellenanyagra pozitív beteg esetén kizárólag a szérumban találtunk pozitivitást, míg az anti-LGI1 és anti-Caspr2 kettős pozitív egyén esetén mindkét mintatípusban azonos erősségű pozitivitást láttunk.

Statisztikai analízisünk során vizsgáltuk az AE-specifikus ellenanyagok nem és életkor szerinti megoszlását. Az anti-NMDAR autoantitestek főleg fiatal nőkre jellemzőek: a 42 anti-NMDAR-pozitív betegből 28 nő volt, medián életkoruk 25 év. Az anti-LGI1 ellenanyagok leggyakrabban középkorú férfiakban fordultak elő: a 9 anti-LGI1-pozitív betegből 6 férfi volt, medián életkoruk 51 év. Az anti-$GABA_BR$ antitesteket elsősorban idősebb férfiakban mértük: a 7 anti-$GABA_BR$-pozitív betegből 4 férfi volt, medián életkoruk 58 év. Az anti-Caspr2 ellenanyagok jellemzően felnőtt férfiakban voltak kimutathatók: a 4 anti-Caspr2-pozitív betegből 3 férfi volt, medián életkoruk 52 év. Két betegben egyidejűleg két különböző autoantitestet mértünk: egyikük egy 3 éves fiú volt, akinél anti-LGI1 és anti-Caspr2 ellenanyagokat mutattunk ki, míg a másik beteg egy 60 éves férfi, akinél anti-$GABA_BR$- és anti-Caspr2-pozitivitást detektáltunk *(3. táblázat).*



2. ÁBRA. Az autoimmun enkefalitisz irányú vizsgálatkérések alakulása a 2012–2018 közötti időszakban. Az oszlopdiagramok a pozitív és negatív eredménnyel zárult vizsgálatokat ábrázolják éves megoszlásban. A teljes időszakra vonatkozó vizsgálatszámok mellett a vizsgált betegekre vonatkozó eredményeinket is feltüntettük

MEGBESZÉLÉS

A klasszikus paraneopláziás neurológiai szindrómák mellett az utóbbi évtizedben különültek el önálló szindrómaként az autoimmun enkefalitiszek, melyek kialakulásáért nem az intracelluláris, hanem a sejtfelszíni/szinaptikus fehérjék ellen képződő ellenanyagok felelősek. Az új autoantitestek felfedezése, melyek változatos idegrendszeri kórképekkel társulnak, diagnosztikus és terápiás szemléletváltáshoz vezetett számos, korábban idiopátiásnak vélt neurológiai szindróma kapcsán. Jelenlegi munkánkban a klasszikus paraneopláziás neurológiai szindrómákhoz, illetve az autoimmun enkefalitiszekhez társuló autoantitestek magyar lakosságra vonatkozó jellegzetességeit vizsgáltuk. Magyarországon az első és eddigi legnagyobb betegszámú retrospektív statisztikai analízis

3. TÁBLÁZAT. Autoimmun enkefalitiszre jellemző autoantitestre pozitív betegek (n=60) megoszlása és jellemzői a 2012–2018-as időszakban

Autoantitest	Betegek száma	Férfi:nő	Életkor (év) medián (tartomány)	Incidencia/100 000 lakos
NMDAR	42	0,5:1	25 (1–75)	0,42
LGI1	9	1:0,5	51 (3–67)	0,09
$GABA_BR$	7	1:0,8	58 (16–81)	0,07
Caspr2	4	1:0,3	52 (3–72)	0,04
AMPAR1	0	–	–	–
AMPAR2	0	–	–	–

Az egyes autoantitestekre pozitív betegek száma, nem és életkor szerinti megoszlása, valamint a betegség adott időszakban való incidenciája látható. Két beteg esetén egyidejűleg két különböző autoantitestet találtunk: az egyik betegnél LGI1 és Caspr2, míg a másiknál $GABA_BR$ és Caspr2 elleni antitestek bizonyultak pozitívnak

keretében 2362 PNS- és 1034 AE-gyanús beteg laboratóriumi teszteredményeinek értékelését végeztük el. A két kórkép kimutatására irányuló vizsgálatkérések száma folyamatosan emelkedő tendenciát mutat. PNS esetén a pozitív eredményű vizsgálatkérések aránya a legmagasabb 2018-ban volt, míg AE esetén 2012-ben volt a legmagasabb a pozitivitás aránya, az utóbbi években alacsonyabb pozitivitást találtunk. A két betegség közötti különbség a pozitivitások megoszlását illetően abból adódhat, hogy laboratóriumunkban AE esetén jelenleg a 6 leggyakoribb autoantitest vizsgálatát végezzük, azonban előfordulhat, hogy az általunk negatívnak talált betegek csoportjában esetleg egy újonnan felfedezett ellenanyag (DPPX, IgLON5, GlyR, mGluR1, mGluR5, neuroexin 3 alfa, neurofaszcin 155, contactin 1) áll a háttérben, mely magyarázhatja az általunk kapott eredményeket. A jövőben ezen újabb autoantitestek kimutatására szolgáló DPPX, IgLON5, GlyR receptorfehérjék génjeivel transzfektált sejt assay-k bevezetését is tervezzük.

Az általunk kapott eredmények a PNS-specifikus ellenanyagok előfordulási gyakoriságát, valamint nem és életkor szerinti megoszlását illetően szintén egybehangzónak bizonyultak a korábbi adatokkal (5). A PNS-betegek esetén a nemek közötti megoszlás tekintetében a nők magasabb arányban voltak érintettek a férfiakhoz képest, és a betegség főleg az idősebb korosztályban jelentkezett. Az általunk vizsgált betegek medián életkora 62 év volt; 120 nő és 70 férfi.

Az általunk kapott adatok az AE-specifikus autoantitestek előfordulási gyakoriságát, valamint nem és életkor szerinti megoszlását illetően a szakirodalommal egybehangzónak bizonyultak. Az anti-NMDAR enkefalitisz bizonyult a leggyakoribbnak, mely elsősorban fiatal nőket érintett. Ezt követte a gyakorisági sorrendben az anti-LGI1 enkefalitisz, mely középkorú férfiakban jelentkezett, majd az anti-$GABA_BR$ enkefalitisz idősebb férfiakban és végül az anti-Caspr2 enkefalitisz felnőtt férfiakban (6).

Fontos hangsúlyozni, hogy a két kórkép esetén a megfelelő és korai diagnózis felállításához eltérő labordiagnosztikai módszerek alkalmazása szükséges. PNS esetén rekombináns antigéneket tartalmazó line-immunoblot assay-t, míg AE esetén az antigéneket magasan expresszáló HEK293 sejteket tartalmazó sejt-alapú indirekt immunfluoreszcens BIOCHIP assay-t alkalmazunk. PNS esetén kizárólag a szérum tesztelése is elegendő az autoantitestek kimutatására, azonban AE esetén a szérum- és likvorminták egyidejű vizsgálata javasolt. A jellegzetes autoantitestek detektálása nagymértékben elősegíti ezen idegrendszeri szindrómák diagnózisát, valamint a terápiás megközelítést egyaránt. PNS esetén az okkult tumor időbeni felismerése és kezelése az elsődleges, az alkalmazott immunterápia ritkán vezet javuláshoz. Ezzel szemben AE esetén az időben megkezdett immunterápia az esetek döntő többségében hatásosnak bizonyul és enyhítheti a betegség lefolyását, elősegítheti a betegek felépülését, valamint csökkentheti egy esetleges későbbi visszaesés kockázatát. A PNS- és AE-gyanús betegek száma folyamatosan emelkedő tendenciát mutat – melyet saját eredményeink is alátámasztottak –, emellett egyre bővül az újonnan felfedezett autoantitestek száma, melyek felelősek a kórképek kialakulásáért. Ezek mind a megbízható labordiagnosztikai módszerek kifejlesztését, illetve bevezetését hangsúlyozzák, melyek lehetővé teszik a pontos és korai diagnózis felállítását (7). A kórképek időbeni felismerése rendkívül fontos, hiszen megfelelő kezelés hiányában akár fatális kimenetelűek is lehetnek.

IRODALOM

1. Csépány T, Illés Zs. Klinikai neuroimmunológia, 2. kiadás. Medical Tribune Scientific Hungary 2014, pp. 275–306
2. Dalmau J, Graus F. Antibody-mediated encephalitis. N Engl J Med 378:840–851, 2018
3. Rosenfeld MR, Dalmau J. Paraneoplastic neurologic syndromes. Neurol Clin 36:675–685, 2018
4. Graus F, Titulaer MJ, Balu R, et al. A clinical approach to diagnosis of autoimmune encephalitis. Lancet Neurol 15:391–404, 2016
5. Graus F, Keime-Guibert F, Reñe R, et al. Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients. Brain 124:1138–1148, 2001
6. Ricken G, Schwaiger C, De Simoni D, et al. Detection methods for autoantibodies in suspected autoimmune encephalitis. Front Neurol 9:841, 2018
7. Naides SJ. The role of the laboratory in the expanding field of neuroimmunology: Autoantibodies to neural targets. J Immunol Methods 463:1–20, 2018

Received: 7 May 2019 | Revised: 6 September 2019 | Accepted: 5 October 2019

DOI: 10.1002/brb3.1454

ORIGINAL RESEARCH

Brain and Behavior Open Access WILEY

Single-center study of autoimmune encephalitis-related autoantibody testing in Hungary

Zsófia Hayden | Katalin Böröcz | Zsuzsanna Csizmadia | Péter Balogh | Zoltán Kellermayer | Kornélia Bodó | József Najbauer | Tímea Berki

Department of Immunology and Biotechnology, Clinical Center, University of Pécs Medical School, Pécs, Hungary

Correspondence
Tímea Berki, Department of Immunology and Biotechnology, Clinical Center, University of Pécs Medical School, Pécs, Hungary.
Email: berki.timea@pte.hu

Funding information
GINOP, Grant/Award Number: 232-15-2016-00050; EFOP, Grant/Award Number: 361-16-2016-00004

Abstract

Objective: Autoantibody detection is crucial for the early diagnosis of autoimmune encephalitis (AIE) since prompt therapy can determine the disease outcome. Here, we report a single-center 6-year retrospective study of autoantibody testing in AIE in the Hungarian population.

Methods: Serum and/or cerebrospinal fluid (CSF) autoantibody tests were performed using cell-based indirect immunofluorescence assay for AIE diagnosis. Samples were provided by neurology clinics as part of a nationwide program. Test results were analyzed for samples received during the period from 2012 to 2018.

Results: We tested 1,247 samples from 1,034 patients with suspected AIE. Autoantibodies were present in 60 patients (5.8% of total). The distribution of patients with different autoantibodies by age and sex was as follows: NMDAR (70%), mostly in young females, LGI1 (15%) in middle-aged males, $GABA_BR$ (12%) in elderly males, and Caspr2 (7%) in males. Long-term follow-up was conducted in 30 patients with repeated test requests, of which 17 remained positive, and 13 switched to negative.

Conclusion: We report the most comprehensive clinical laboratory study of autoantibody testing in AIE in the Hungarian population. Our results show that the frequency of different autoantibody types in AIE corresponds to the data described in the literature.

KEYWORDS

autoantibodies, autoimmune encephalitis, biochip, laboratory diagnostics

1 | INTRODUCTION

During the past few years, it has been recognized that there are central nervous system (CNS) disorders presenting in the form of limbic encephalitis, in which the presence of autoantibodies against the neuronal cell surface receptors such as NMDAR, $GABA_BR$, and AMPAR or synaptic proteins, LGI1 and Caspr2, has been documented and shown to be responsible for the development of the symptoms (Dalmau, Geis, & Graus, 2017; Dalmau & Graus, 2018; Newman et al., 2016). The target molecules of these autoantibodies play important roles in synaptic signal transmission and neuronal plasticity. The autoimmune reaction to these antigens in the majority of cases leads to epileptic seizures and neuropsychiatric symptoms (Table 1) (Celicanin et al., 2017; Fukata, Yokoi, & Fukata, 2018; Honnorat & Plazat, 2018; van Sonderen, Petit-Pedrol, Dalmau, & Titulaer, 2017; Szots et al., 2017). In autoimmune encephalitis (AIE),

the autoantibodies bind to the extracellular epitopes of the neuronal cell surface receptors or their associated proteins, which can lead to alteration of the structure and function of target antigens by different mechanisms. Thus in anti-NMDAR encephalitis, autoantibodies induce receptor cross-linking and internalization, in anti-LGI1 encephalitis autoantibodies interfere with protein–protein interactions, and in anti-$GABA_BR$ encephalitis autoantibodies may block the function of the target antigen (Hughes et al., 2010; Ohkawa et al., 2013). The autoantibodies cause reversible neuronal dysfunction, and immunotherapy (e.g., steroids, plasmapheresis, immunosuppression, and intravenous immunoglobulin) results in reduction of autoantibody levels and can lead to the improvement of patients (Hermetter, Fazekas, & Hochmeister, 2018). Patients can have a fatal outcome in case of lack of the proper therapy. This highlights the importance of early clinical diagnosis of AIE, in which the laboratory has a crucial role by providing accurate and reproducible testing of serum and/or cerebrospinal fluid (CSF) samples for the presence of autoantibodies.

2 | MATERIALS AND METHODS

2.1 | Samples

We carried out a retrospective statistical study of the results obtained by our laboratory based on serum and CSF analysis of patients with suspected AIE. Our laboratory was the first to introduce these tests in Hungary and has received samples from various neurological clinics and hospitals from 2012 through 2018 as part of a nationwide program. Serum and CSF samples were obtained with patients' informed consent. The study was approved by Regional Research Ethics Committee of the Medical Center, University of Pécs (RIKEB 6966/2017).

2.2 | Detection of AIE autoantibodies

For detection of AIE-related autoantibodies, a cell-based indirect immunofluorescence BIOCHIP assay was used (Euroimmun, Autoimmune Encephalitis Mosaic 1, FA 112d-1003-1). On the BIOCHIP slide, HEK293 cells expressing six different antigens of interest (NMDAR, LGI1, Caspr2, $GABA_BR$, AMPAR1, and AMPAR2) are immobilized as a mosaic. Five samples can be investigated on a single slide; one mosaic is suitable for the detection of six types of autoantibodies. Optimization of the assay was based on the recommended protocol included in the Manufacturer's Instruction. About 30 µl of the samples (sera diluted 1:10 or CSF undiluted) were incubated on the BIOCHIP containing the six transfected cell lines for 30 min at room temperature, followed by two washing steps with PBS Tween-20 buffer (included in the kit) for 5 min. For secondary labeling, 25 µl of anti-Human IgG (Fc-specific)-FITC antibody specifically recognizing Fc fragment of all human IgG subclasses (IgG is the most frequently associated immunoglobulin isotype in AIE (Ricken et al., 2018)), included in the kit, was applied for 30 min at room

TABLE 1 Main characteristics of different autoimmune encephalitis types

Autoantibody	Clinical features	MRI (T2/FLAIR)	Tumor	Prognosis	Male/Female	Median age (years)
NMDAR	Prodromal stage (fever, headache, abdominal pain) Psychiatric symptoms (agitation, hallucinations, delusions, catatonia, psychosis) Later manifestations (reduction of speech, memory deficit, orofacial and limb dyskinesias, seizures, decreased level of consciousness, autonomic instability)	Normal or nonspecific changes	58%, (age- and sex-dependent) in young women ovarian teratoma	81% have a good outcome	1:4	21
LGI1	Faciobrachial dystonic seizures, limbic encephalitis, hyponatremia, sleep disorders, memory, and cognitive deficits	Hyperintense signal in medial temporal lobes	<5%, thymoma	70% have a good outcome	2:1	64
Caspr2	Neuromyotonia, Morvan's syndrome, limbic encephalitis, insomnia, neuropathic pain	Hyperintense signal in medial temporal lobes	<5%, thymoma	70% have a good outcome	9:1	66
$GABA_BR$	Limbic encephalitis, seizures Rarely: cerebellar ataxia, opsoclonus-myoclonus	Hyperintense signal in medial temporal lobes	50%, SCLC	80% initially good response but have poor prognosis due to SCLC	1.5:1	61
AMPAR	Limbic encephalitis, seizures Rarely: psychiatric symptoms	Hyperintense signal in medial temporal lobes	56%, SCLC, thymoma, or breast carcinoma	70% have a good outcome	1:2.3	56

Note: Based on Dalmau and Graus (2018), Newman et al. (2016), van Sonderen et al. (2017).

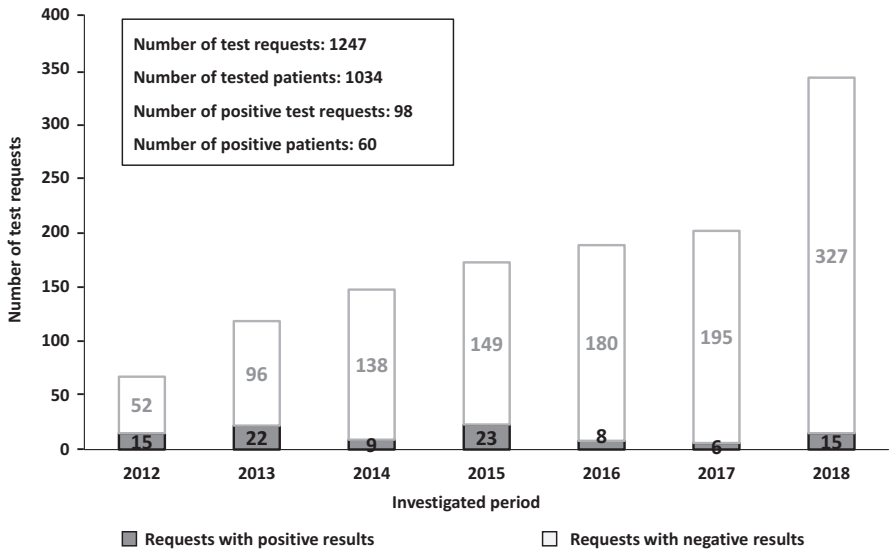


FIGURE 1 Annual distribution of autoimmune encephalitis test requests. The cell-based indirect immunofluorescence BIOCHIP assay was introduced at our laboratory in 2012, and test results were analyzed through 2018. The number of positive tests varied in time, although the total number of requests increased each year. Data of 12 equivocal test results are not shown

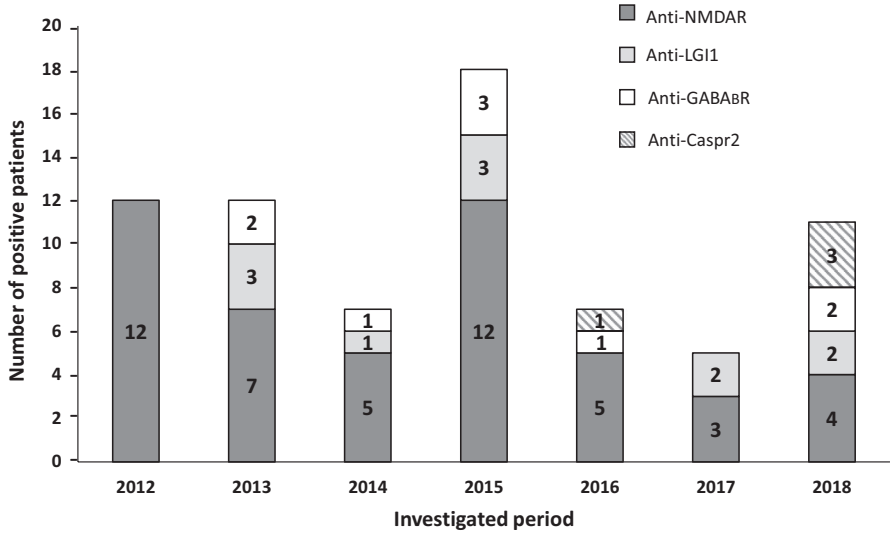


FIGURE 2 Annual distribution of the positively detected autoantibody types in autoimmune encephalitis patients. The ratio of autoantibody-positive patients in decreasing order was NMDAR > LGI1 > $GABA_BR$ > Caspr2. No patients were found with anti-AMPAR1 or anti-AMPAR2 autoantibody positivity

temperature. After two washes for 5 min, glycerol (included in the kit) was used for covering the slides. Positive and negative controls were used to help evaluate the patient samples. The advantage of this test is the capacity of detecting simultaneously the presence of six different types of autoantibodies in a single sample. In case of positive or equivocal test results for anti-NMDAR autoantibodies, the anti-Glutamate Receptor (type NMDA) IIFT kit was used as a confirmatory test (Euroimmune). These BIOCHIP slides contain NMDAR expressing and control HEK293 cells immobilized as a mosaic. Optimization was also based on the Manufacturer's Instruction.

2.3 | Fluorescence imaging and evaluation

Fluoresce imaging was performed using a fluorescence microscope (Olympus BX61) coupled with Zeiss Axiocam 305 color microscope digital camera and image processing system. The BIOCHIPs were evaluated independently by at least two laboratory specialists. Positive and negative controls were used, and reactions were graded as strong positive, positive, low positive, equivocal, and negative.

3 | RESULTS

3.1 | Annual distribution and frequency of autoimmune encephalitis-related autoantibody types

Since the introduction of tests for AIE in 2012 at our institution, the number of test requests for diagnosing the disease has increased each year. Our laboratory has received 1,247 test requests (sera and/or CSF samples) from a total of 1,034 patients for detection of AIE-related autoantibodies (Figure 1). We employed a cell-based indirect immunofluorescence BIOCHIP assay for the detection of six ion channel or their associated protein-specific autoantibodies (NMDAR, Caspr2, $GABA_BR$, AMPAR1, AMPAR2, and LGI1). We have found 98 positive samples belonging to 60 patients. This result reflects that autoantibodies were present in only 5.8% of the patients with clinically suspected AIE. The frequency of the positive samples varied with the examination period; the highest ratio of positive test requests was during the first 4 years after the introduction of the test [22.4% (2012), 18.7% (2013), 6.1% (2014), 13.4% (2015)].

Autoantibody	Number of patients (%)	Male/Female	Median age/range (years)	Incidence per 100,000 Person-Years
NMDAR	42 (70)	0.5:1	25 (1-75)	0.42
LGI1	9 (15)	1:0.6	51 (3-67)	0.09
GABAʙR	7 (12)	1:0.75	58 (16-81)	0.07
Caspr2	4 (7)	1:0	52 (3-72)	0.04
AMPAR1	0	–	–	–
AMPAR2	0	–	–	–

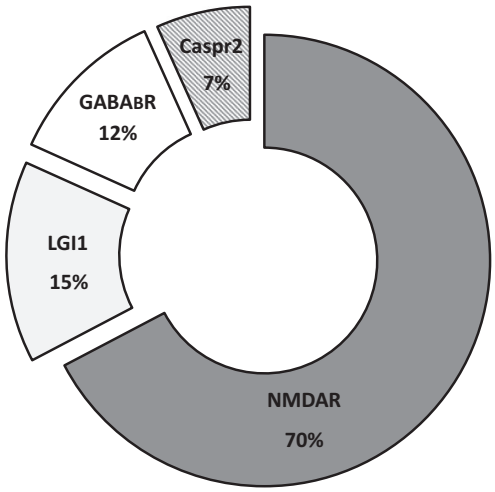


FIGURE 3 Frequency and distribution of autoantibody types by age and sex in autoimmune encephalitis patients. The inserted table shows the number of patients belonging to the different AIE autoantibody types. Anti-NMDAR autoantibodies were most frequently detected in young women, anti-LGI1 autoantibodies occurred in middle-aged males, anti-$GABA_BR$ autoantibodies were present in elderly males, and anti-Caspr2 autoantibodies affected adult males

Although the number of test requests continued to increase, the ratio of positivity was lower during the past 3 years [4.3% (2016), 3% (2017), 4.4% (2018)]. The highest proportion of positive tests for AIE was 22.4% (2012), and the lowest was 3% (2017) (Figure 1). By analyzing the annual distribution of patients with positive AIE test results, we found marked differences. The number of newly diagnosed patients was 3–14/year, while requests with positive results of the already diagnosed positive patients were only 1–4/year (data not shown). Figure 2 shows the annual distribution of positive patients with different types of autoantibodies. Patients with anti-NMDAR antibodies showed the highest frequency for each year during the examined period. The frequency of different types of autoantibodies also varied: anti-NMDAR autoantibody was present in 70%, anti-LGI1 in 15%, anti-$GABA_BR$ in 12%, and anti-Caspr2 in 7% of patients (Figure 3). Two patients showed positivity for two types of autoantibodies simultaneously (one patient showed positivity against LGI1 and Caspr2, and the other against $GABA_BR$ and Caspr2). In 12 patients, the results obtained from sera were equivocal (11 NMDAR and one $GABA_BR$); of which, five patients were negative upon simultaneous testing of CSF, and new samples from three patients were negative upon retesting.

3.2 | Characteristics of patients with positive autoantibodies

We investigated the distribution of AIE-related autoantibody subtypes by age and sex (Figure 3). Anti-NMDAR encephalitis mostly affected females: of the 42 anti-NMDAR-positive patients, 28 were female, with median age of 25 years. Anti-LGI1 encephalitis most frequently occurred in middle-aged males: of the nine anti-LGI1-positive patients, six were male, with median age of 51 years. Anti-$GABA_BR$ encephalitis affected elderly males: of the seven anti-$GABA_BR$-positive patients, four were male, with median age of 58 years. Anti-Caspr2 encephalitis occurred in male adults: of the four anti-Caspr2-positive patients, three were male, with median age of 52 years (Figure 3). Two patients showed positivity for two types of autoantibodies simultaneously: a 3-year-old boy was positive for both anti-LGI1 and anti-Caspr2, and a 60-year-old male was positive for anti-$GABA_BR$ and anti-Caspr2. Of the total 60 positive patients, in 30 cases repeated tests were performed at different time points (Table 2). In 17 cases, repeated laboratory tests resulted in autoantibody positivity multiple times, while in 13 patients the original positive autoantibody result subsequently switched to negative. Five patients (one anti-NMDAR, one anti-LGI1, one anti-$GABA_BR$, and one positive for both anti-LGI1 and anti-Caspr2 antibodies) became negative within 1 year after the first positive test; four anti-NMDAR-positive patients switched to negative within the 2nd year after the first positive test; three patients (two anti-NMDAR and one anti-LGI1) turned to negative within the 3rd year; and one anti-NMDAR patient was found negative during the 5th year after the first positive test (Table 2).

3.3 | Influence of sample type on the laboratory test results

Among the 60 positive patients, in 34 cases (57% of autoantibody-positive patients) autoantibodies were detected only in serum or only in CSF samples. In 28 cases (47%) only serum, while in six cases (10%) only CSF was tested. In 26 cases (43%), both sample types were investigated. Analyzing in more detail this group of autoantibody-positive patients, we found marked differences regarding the sample type and the strength of detected positivity in the different AIE-related autoantibody types. Among 17 anti-NMDAR patients—whose sera and CSF were tested simultaneously—in seven cases autoantibodies were detected in both serum and CSF, but in three cases stronger positivity was detected in the CSF. In seven anti-NMDAR patients, positivity was found only in CSF and in three cases only in serum. In three anti-LGI1 patients, we detected

TABLE 2 Groups of autoantibody-positive patients with repeated test requests

Patients	ID	Autoantibody type	Follow-up time (years)	Number of test requests
Repeatedly positive	1.	NMDAR	1 (2013)	7
	2.		5 (2014–2018)	3
	3.		2 (2013–2014)	2
	4.		1 (2015)	2
	5.		2 (2015–2016)	2
	6.		1 (2012)	2
	7.		1 (2012)	2
	8.		4 (2012–2015)	2
	9.		1 (2018)	2
	10.		1 (2015)	2
	11.		2 (2016–2017)	2
	12.		1 (2015)	2
	13.		1 (2015)	2
	14.	LGI1	3 (2013–2015)	4
	15.	$GABA_BR$	3 (2013–2015)	3
	16.		1 (2015)	2
	17.		1 (2018)	2
Switched to negative	1.	NMDAR	5 (2012–2016)	7
	2.		3 (2016–2018)	4
	3.		2 (2012–2013)	2
	4.		1 (2017)	2
	5.		2 (2012–2013)	2
	6.		2 (2012–2013)	2
	7.		2 (2012–2013)	2
	8.		3 (2012–2014)	2
	9.	LGI1	1 (2013)	2
	10.		1 (2018)	2
	11.		3 (2013–2015)	3
	12.	LGI1 + Caspr2	1 (2018)	3
	13.	$GABA_BR$	1 (2015)	2

autoantibodies in sera only, but not in the CSF. In four anti-$GABA_BR$ patients, positivity was detected in both serum and CSF, and in one case, serum showed higher level of antibodies than CSF. In one anti-Caspr2 patient, only the serum was positive. In one patient, both anti-LGI1 and anti-Caspr2 antibodies were detected in both sample types, although the anti-Caspr2 positivity was stronger in the serum than in CSF (Table 3).

4 | DISCUSSION

AIE has been recognized during the past decade as a distinct disease entity (Dalmau & Graus, 2018; Venkatesan, Michael, Probasco, Geocadin, & Solomon, 2019). The discovery of AIE subtypes has changed the diagnostic and therapeutic approaches to many neurological disorders previously considered to be idiopathic. Our aim was to investigate the characteristics of autoantibody testing in patients with AIE, in which early and accurate clinical diagnosis plays a pivotal role. The current retrospective analysis included laboratory test results from 1,034 patients with suspected AIE, making it the most comprehensive study in Hungary to date. Our data confirmed the relative prevalence of AIE subtypes described previously (Dalmau & Graus, 2018). Anti-NMDAR encephalitis was the most common subtype, followed by anti-LGI1, anti-$GABA_BR$, and anti-Caspr2 encephalitis, which is in agreement with previous reports (Gable, Sheriff, Dalmau, Tilley, & Glaser, 2012; van Sonderen et al., 2017). Our data regarding the age and sex of AIE patients agree with the data published in the literature (Ricken et al., 2018).

Highly sensitive and specific multiplex cell-based assay is available for AIE diagnostics, in which HEK293 cells expressing high levels of antigens of interest are used. Analysis of serum and

TABLE 3 Summary of test results of autoimmune encephalitis-related autoantibodies in patients with serum and/or CSF positivity according to AIE subtypes

Patient ID	Serum	CSF	Autoantibody subtype
1.	+	NA	NMDAR
2.	+	+	
3.	+	NA	
4.	NA	+	
5.	+	NA	
6.	+	NA	
7.	+	NA	
8.	+	NA	
9.	+	NA	
10.	+	NA	
11.	+	+	
12.	+	NA	
13.	+	+	
14.	+	NA	
15.	+	NA	
16.	+	NA	
17.	+	+	
18.	+	+++	
19.	+	NA	
20.	–	+	
21.	NA	+	
22.	–	+	
23.	+	+++	
24.	–	+	
25.	+	NA	
26.	+/–	+	
27.	–	+	
28.	+	NA	
29.	–	+	
30.	NA	+	
31.	NA	+++	
32.	NA	+	
33.	+	++	
34.	+	NA	
35.	++	NA	
36.	NA	+	
37.	+	–	
38.	+	NA	
39.	+	–	
40.	–	++	
41.	+	–	
42.	++	NA	
43.	+	–	LGI1
44.	+	NA	
45.	+	NA	
46.	+	NA	
47.	++	NA	
48.	+	–	
49.	+++	NA	
50.	++	–	
51.	+	+	LGI1 and Caspr2
	++	+	
52.	+	+	$GABA_BR$
53.	+	+	
54.	+	+	
55.	+	NA	
56.	++	NA	
57.	++	+	
58.	+	NA	$GABA_BR$ and Caspr2
	+	NA	
59.	+	NA	Caspr2
60.	+	–	

Abbreviations: –, negative; +, low positive; +/–, equivocal; ++, positive; +++, strong positive; NA, not available.

CSF samples of patients with AIE suggested that both types of samples should be tested, especially in patients with anti-NMDAR autoantibodies, since in most patients the autoantibodies are detected in the CSF, while the serum might be negative. However, in some anti-LGI1 antibody-positive patients, autoantibodies can be found only in serum or only in CSF (Table 3) (Dalmau & Graus, 2018). It has been reported previously that detection of the characteristic autoantibodies in clinically suspected AIE could serve as confirmatory diagnosis, and in case of anti-NMDAR encephalitis, testing of CSF can be used to monitor disease activity, and autoantibody levels often correlate with patient outcome and relapse rates (Lee & Lee, 2016; Wandinger, Leypoldt, & Junker, 2018). The highest sensitivity and specificity of the tests can be achieved by testing both serum and CSF. It should be noted, however, that false-positive results can occur more commonly when serum samples are tested.

5 | CONCLUSION

In conclusion, our data are in agreement with previous reports on the frequency and distribution of AIE-related autoantibodies, and detection of which can significantly aid the diagnosis of AIE and suggests treatment strategies. Early immunotherapy is often effective and can reduce the severity of AIE, promote recovery and decrease the risk of relapse (Crisp, Kullmann, & Vincent, 2016; Dalmau & Graus, 2018; Ricken et al., 2018; Varley, Taylor, & Irani, 2018). As the number of patients affected by AIE is increasing and the spectrum of the newly identified autoantibodies broadens, it is important to employ reliable laboratory tests that allow accurate diagnosis to be made. The evaluation of patients with suspected autoimmune encephalitis should include testing for autoantibodies in both serum and CSF simultaneously, since some autoantibodies can be preferentially found only in serum or in CSF (Dalmau & Graus, 2018). Finally, early recognition of AIE subtypes is important because without proper treatment they can have fatal outcome.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

ZH, KaB, ZC, PB, ZK, and TB performed the tests and evaluated the results. ZH, KaB, and TB analyzed data. ZH and KoB performed imaging of indirect immunofluorescence staining of BIOCHIP using an Olympus BX61 fluorescence microscope. ZH, JN, and TB wrote the paper with editorial help from PB and ZK.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Zsófia Hayden https://orcid.org/0000-0003-2565-1144

REFERENCES

Celicanin, M., Blaabjerg, M., Maersk-Moller, C., Beniczky, S., Marner, L., Thomsen, C., … Pinborg, L. H. (2017). Autoimmune encephalitis associated with voltage-gated potassium channels-complex and leucine-rich glioma-inactivated 1 antibodies – A national cohort study. *European Journal of Neurology*, *24*(8), 999–1005. https://doi.org/10.1111/ene.13324

Crisp, S. J., Kullmann, D. M., & Vincent, A. (2016). Autoimmune synaptopathies. *Nature Reviews Neuroscience*, *17*(2), 103–117. https://doi.org/10.1038/nrn.2015.27

Dalmau, J., Geis, C., & Graus, F. (2017). Autoantibodies to synaptic receptors and neuronal cell surface proteins in autoimmune diseases of the central nervous system. *Physiological Reviews*, *97*(2), 839–887. https://doi.org/10.1152/physrev.00010.2016

Dalmau, J., & Graus, F. (2018). Antibody-mediated encephalitis. *New England Journal of Medicine*, *378*(9), 840–851. https://doi.org/10.1056/NEJMra1708712

Fukata, M., Yokoi, N., & Fukata, Y. (2018). Neurobiology of autoimmune encephalitis. *Current Opinion in Neurobiology*, *48*, 1–8. https://doi.org/10.1016/j.conb.2017.07.012

Gable, M. S., Sheriff, H., Dalmau, J., Tilley, D. H., & Glaser, C. A. (2012). The frequency of autoimmune N-methyl-D-aspartate receptor encephalitis surpasses that of individual viral etiologies in young individuals enrolled in the California Encephalitis Project. *Clinical Infectious Diseases*, *54*(7), 899–904. https://doi.org/10.1093/cid/cir1038

Hermetter, C., Fazekas, F., & Hochmeister, S. (2018). Systematic review: Syndromes, early diagnosis, and treatment in autoimmune encephalitis. *Frontiers in Neurology*, *9*, 706. https://doi.org/10.3389/fneur.2018.00706

Honnorat, J., & Plazat, L. O. (2018). Autoimmune encephalitis and psychiatric disorders. *Revue Neurologique*, *174*(4), 228–236. https://doi.org/10.1016/j.neurol.2017.11.004

Hughes, E. G., Peng, X., Gleichman, A. J., Lai, M., Zhou, L., Tsou, R., … Balice-Gordon, R. J. (2010). Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. *Journal of Neuroscience*, *30*(17), 5866–5875. https://doi.org/10.1523/JNEUROSCI.0167-10.2010

Lee, S. K., & Lee, S. T. (2016). The laboratory diagnosis of autoimmune encephalitis. *Journal of Epilepsy Research*, *6*(2), 45–50. https://doi.org/10.14581/jer.16010

Newman, M. P., Blum, S., Wong, R. C., Scott, J. G., Prain, K., Wilson, R. J., & Gillis, D. (2016). Autoimmune encephalitis. *Internal Medicine Journal*, *46*(2), 148–157. https://doi.org/10.1111/imj.12974

Ohkawa, T., Fukata, Y., Yamasaki, M., Miyazaki, T., Yokoi, N., Takashima, H., … Fukata, M. (2013). Autoantibodies to epilepsy-related LGI1 in limbic encephalitis neutralize LGI1-ADAM22 interaction and reduce synaptic AMPA receptors. *Journal of Neuroscience*, *33*(46), 18161–18174. https://doi.org/10.1523/JNEUROSCI.3506-13.2013

Ricken, G., Schwaiger, C., De>Simoni, D., Pichler, V., Lang, J., Glatter, S., … Höftberger, R. (2018). Detection methods for autoantibodies in suspected autoimmune encephalitis. *Frontiers in Neurology*, *9*, 841. https://doi.org/10.3389/fneur.2018.00841

Szots, M., Blaabjerg, M., Orsi, G., Iversen, P., Kondziella, D., Madsen, C. G., … Illes, Z. (2017). Global brain atrophy and metabolic dysfunction in LGI1 encephalitis: A prospective multimodal MRI study. *Journal of the Neurological Sciences*, *376*, 159–165. https://doi.org/10.1016/j.jns.2017.03.020

van Sonderen, A., Petit-Pedrol, M., Dalmau, J., & Titulaer, M. J. (2017). The value of LGI1, Caspr2 and voltage-gated potassium channel antibodies in encephalitis. *Nature Reviews. Neurology*, *13*(5), 290–301. https://doi.org/10.1038/nrneurol.2017.43

Varley, J., Taylor, J., & Irani, S. R. (2018). Autoantibody-mediated diseases of the CNS: Structure, dysfunction and therapy. *Neuropharmacology*, *132*, 71–82. https://doi.org/10.1016/j.neuropharm.2017.04.046

Venkatesan, A., Michael, B. D., Probasco, J. C., Geocadin, R. G., & Solomon, T. (2019). Acute encephalitis in immunocompetent adults. *Lancet*, *393*(10172), 702–716. https://doi.org/10.1016/S0140-6736(18)32526-1

Wandinger, K. P., Leypoldt, F., & Junker, R. (2018). Autoantibody-mediated encephalitis. *Deutsches Ärzteblatt International*, *115*(40), 666–673. https://doi.org/10.3238/arztebl.2018.0666

How to cite this article: Hayden Z, Böröcz K, Csizmadia Z, et al. Single-center study of autoimmune encephalitis-related autoantibody testing in Hungary. *Brain Behav.* 2019;9:e01454. https://doi.org/10.1002/brb3.1454

frontiers in Neurology

ORIGINAL RESEARCH
published: 09 March 2021
doi: 10.3389/fneur.2021.611597

Clinical Characteristics and Outcome of Neuronal Surface Antibody-Mediated Autoimmune Encephalitis Patients in a National Cohort

Zsófia Hayden[1], Beáta Bóné[2], Gergely Orsi[3,4], Monika Szots[5], Ferenc Nagy[5], Tünde Csépány[6], Zsolt Mezei[7], Cecília Rajda[8], Diána Simon[1], József Najbauer[1], Zsolt Illes[9,10] and Timea Berki[1]*

[1] Department of Immunology and Biotechnology, Clinical Center, University of Pécs Medical School, Pécs, Hungary, [2] Department of Neurology, University of Pécs, Pécs, Hungary, [3] MTA-PTE Clinical Neuroscience MR Research Group, Pécs, Hungary, [4] Department of Neurosurgery, Clinical Centre, University of Pécs Medical School, Pécs, Hungary, [5] Department of Neurology, Somogy County Kaposi Mór University Teaching Hospital, Kaposvár, Hungary, [6] Department of Neurology, University of Debrecen, Debrecen, Hungary, [7] Department of Neurology, Semmelweis University, Budapest, Hungary, [8] Department of Neurology, University of Szeged, Szeged, Hungary, [9] Department of Neurology, Odense University Hospital, Odense, Denmark, [10] Institute of Clinical Research, BRIDGE, University of Southern Denmark, Odense, Denmark

OPEN ACCESS

Edited by:
Long-Jun Wu,
Mayo Clinic, United States

Reviewed by:
Gregory S. Day,
Mayo Clinic Florida, United States
Christian G. Bien,
Mara Hospital, Germany
Sudarshini Ramanathan,
The University of Sydney, Australia

***Correspondence:**
Zsófia Hayden
hayden.zsofia@pte.hu

Specialty section:
This article was submitted to Multiple Sclerosis and Neuroimmunology, a section of the journal Frontiers in Neurology

Received: *29 September 2020*
Accepted: *16 February 2021*
Published: *09 March 2021*

Citation:
Hayden Z, Bóné B, Orsi G, Szots M, Nagy F, Csépány T, Mezei Z, Rajda C, Simon D, Najbauer J, Illes Z and Berki T (2021) Clinical Characteristics and Outcome of Neuronal Surface Antibody-Mediated Autoimmune Encephalitis Patients in a National Cohort. Front. Neurol. 12:611597. doi: 10.3389/fneur.2021.611597

Background: In our previous single-center study of autoimmune encephalitis (AE) related autoantibody test results we found positivity in 60 patients out of 1,034 with suspected AE from 2012 through 2018 as part of a Hungarian nationwide program. In our current multicenter retrospective study, we analyzed the clinical characteristics and outcome of AE patients with positive neuronal cell surface autoantibody test results.

Methods: A standard online questionnaire was used to collect demographic and clinical characteristics, laboratory and imaging data, therapy and prognosis of 30 definitive AE patients in four major clinical centers of the region.

Results: In our study, 19 patients were positive for anti-NMDAR (63%), 6 patients (20%) for anti-LGI1, 3 patients for anti-GABABR (10%) and 3 patients for anti-Caspr2 (10%) autoantibodies. Most common prodromal symptoms were fever or flu-like symptoms (10/30, 33%). Main clinical features included psychiatric symptoms (83%), epileptic seizures (73%) and memory loss (50%). 19 patients (63%) presented with signs of central nervous system (CNS) inflammation, which occurred more frequently in elder individuals ($p = 0.024$), although no significant differences were observed in sex, tumor association, time to diagnosis, prognosis and immunotherapy compared to AE patients without CNS inflammatory markers. Anti-NMDAR encephalitis patients were in more severe condition at the disease onset ($p = 0.028$), although no significant correlation between mRS score, age, sex and immunotherapy was found. 27% of patients ($n = 8$) with associated tumors had worse outcome ($p = 0.045$) than patients without tumor. In most cases, immunotherapy led to clinical improvement of AE patients (80%) who achieved a good outcome (mRS ≤ 2; median follow-up 33 months).

Conclusion: Our study confirms previous publications describing characteristics of AE patients, however, differences were observed in anti-NMDAR encephalitis that showed no association with ovarian teratoma and occurred more frequently among young males. One-third of AE patients lacked signs of inflammation in both CSF and brain MRI, which emphasizes the importance of clinical symptoms and autoantibody testing in diagnostic workflow for early introduction of immunotherapy, which can lead to favorable outcome in AE patients.

Keywords: autoimmune encephalitis, neuronal surface antibody, clinical characteristics, immunotherapy, prognosis

INTRODUCTION

Autoimmune encephalitis (AE) is increasingly recognized as one of the most frequent causes of non-infectious encephalitis (1, 2). In AE, the most common autoantibodies against neuronal cell surface receptors and synaptic proteins include those against NMDAR (N-methyl-D-aspartate receptor), GABABR (γ-aminobutyric acid receptor-B), AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and against proteins associated with the VGKC (voltage-gated potassium channel complex), such as LGI1 (leucine-rich, glioma inactivated 1) and Caspr2 (contactin-associated protein-like 2) (3, 4). AE may present with a wide spectrum of clinical symptoms, such as behavioral and psychiatric disorders, cognitive impairment, changes in the level of consciousness, seizures and movement disorders (5, 6). Besides clinical features suggestive of AE, accurate diagnosis relies on the detection of characteristic autoantibodies in the serum and/or cerebrospinal fluid (CSF), accompanied with auxiliary examinations, such as structural magnetic resonance imaging (MRI) and electroencephalography (EEG) (7). The diagnosis of AE may be complicated as results of cerebral imaging and CSF analysis are often unremarkable, especially in the elderly with autoantibodies against LGI1 and Caspr2 (8, 9). In AE, early introduction of proper therapy is associated with a more favorable outcome, emphasizing the importance of early diagnosis based on clinical symptoms and paraclinical tests at the time of presentation. In our previous single-center study of patients with suspected AE, we found positive autoantibody test results in 60 out of 1,034 patients from 2012 through 2018 as part of a Hungarian nationwide program (10). In that study, long-term (1–5 years) autoantibody testing was conducted in 30 patients with positive autoantibody results, of which 17 remained positive at different time points. The aim of the present retrospective study was to characterize the clinical features and outcome of AE, based on the results of laboratory and paraclinical tests, in a multicenter cohort of Hungarian patients, involving four major clinical centers of the region to collect data of 35 patients with AE-related autoantibody positivity.

MATERIALS AND METHODS

Patient Inclusion

In our current study, we retrospectively identified 35 patients with positive neuronal cell surface autoantibody (NMDAR, LGI1, GABABR, Caspr2). Patients were selected based on our previous observational cohort study of patients who tested positive for at least one neuronal cell surface autoantibody in sera, CSF or both in sera and CSF at the Department of Immunology and Biotechnology, Clinical Center, University of Pécs Medical School, Pécs, Hungary, from January 2012 until December 2019 (10). Of the total 35 patients, 24 were tested in CSF-serum pairs (68.6%), 9 for serum only (25.7%) and 2 for CSF only (5.7%). Five patients with positive antibody results were excluded from further clinical analysis because of alternative diagnosis. Two patients with anti-Caspr2 positivity detected in sera but not in CSF, and one patient with anti-NMDAR positivity in CSF but not in serum had a final diagnosis of stroke (of which the latter patient died). One patient with anti-NMDAR positivity found in sera but not in CSF, and one patient with anti-Caspr2 positivity detected only in sera (CSF was not available) had a final diagnosis of multiple sclerosis. Patients with positive neuronal cell surface autoantibody tests enrolled in this study fulfilled the criteria for definite AE defined by Graus et al. (7): (1) subacute onset (rapid progression < 3 months) of working memory deficits (short-term memory loss), altered mental status, or psychiatric symptoms, and (2) new focal central nervous system (CNS) findings, seizures not explained by a previously known seizure disorder, CSF pleocytosis (white blood cell count > 5 cells/mm^3), or MRI features suggestive of encephalitis, and (3) reasonable exclusion of alternative causes. All anti-NMDAR autoantibody positive patients included in the study had ≥ 2 positive results during confirmatory tests (Euroimmun, The anti-Glutamate Receptor (type NMDA) IIFT kit, FC112d1005-51).

Neuronal Cell Surface Antibody Detection

The antibody panel included anti-NMDAR, anti-GABABR, anti-AMPAR1, anti-AMPAR2, anti-LGI1 and anti-Caspr2. AE-related antibody testing was performed in the routine laboratory, using indirect immunofluorescence (IIFT) BIOCHIP assay with HEK293 cells expressing the genes of the six different proteins (Euroimmun, Autoimmune Encephalitis Mosaic 1, FA 112d-1003-1). The assay was optimized following the guideline included in the Manufacturer's Instruction. The anti-Glutamate Receptor (type NMDA) IIFT kit with BIOCHIP slides containing fields of NMDAR expressing and control HEK293 cells was used as a confirmatory test (Euroimmun, The anti-Glutamate Receptor (type NMDA) IIFT kit, FC112d1005-51) in case of samples positive or equivocal for anti-NMDAR autoantibodies. For histological localization of autoantibody positivity a rat

brain biochip was used for indirect immunofluorescence imaging (Euroimmun, IIFT: Glutamate Receptor Mosaic 3, FA 111m-1003-3).

Data Collection and Analyses

Clinical data were collected using an online questionnaire in collaboration with neurologists specialized in neuroimmunology from four clinical centers in Hungary. Questions about demographics, prodromal symptoms, clinical features, CSF findings, EEG and brain MRI descriptions, therapy and prognosis were included in the questionnaire.

We used the modified Rankin scale (mRS) to measure neurological outcome in AE patients (11–14). The mRS score was determined at onset, at the time of the worst status of the patient, and at the last visit. The mRS score of 0-2 at the last visit was considered as good outcome and > 2 as poor outcome (13). Complete recovery was assessed in AE patients with mRS score of 0 at the last visit following treatment (median: 33 months; range: 1–77) (12). Relapse was defined as the new onset or worsening of symptoms, after at least 2 months of improvement or stabilization (15).

Statistical Methods

Statistical evaluation was performed with the SPSS IBM version 26 (IBM, Armonk, NY, USA). Categorical variables were described as percentages and numerical variables were described as medians and ranges. Mann-Whitney U test for continuous variables and Fisher's exact test for categorical variables were used as appropriate. P-values < 0.05 were considered statistically significant.

Standard Protocol Approvals, Registrations, and Patient Consents

Clinical samples were obtained with patients' informed consent. The study was approved by the Ethics Committee of the Medical Research Council of Hungary (number of approval: 49709-2/2019/EKU).

RESULTS

Demographic Features and Characteristics of AE Patients

We identified and collected detailed clinical data of 30 patients with the diagnosis of definite AE. Of the 30 patients, autoantibodies were tested in CSF-serum pairs in 66.7%, only in serum in 26.7% and only in CSF in 6.7% of patients (**Table 1**). The most common antibody was anti-NMDAR (19/30, 63.3%), followed by anti-LGI1 (6/30, 20%), anti-GABABR (3/30, 10%) and anti-Caspr2 (3/30, 10%). One patient showed positivity for both anti-LGI1 and anti-Caspr2 antibodies. In the anti-NMDAR patient group, autoantibodies were detected in sera and CSF simultaneously in 31.6%, only in CSF in 36.8% and only in sera in 31.6% of patients. All anti-NMDAR autoantibody positive patients had $\geq$2 positive results during confirmatory tests (Euroimmun, The anti-Glutamate Receptor (type NMDA) IIFT kit, FC112d1005-51). In the anti-LGI1 and in the anti-Caspr2 positive patients, in all cases autoantibodies were present only in sera, but not in the CSF with the exception of one patient with anti-LGI1 and anti-Caspr2 antibody positivity detected in both sera and CSF. In all anti-GABABR positive patients, we detected antibodies both in sera and CSF (**Table 1**). The group of 30 patients with definite AE, included 19 men (63.3%) and 11 women (36.7%) with a median age of 39.3 years (range: 1–75 years). Different sex ratios and median age were observed in the different AE types (**Table 1**).

Clinical Features of AE Patients

Median time to diagnosis (onset of clinical symptoms until positive autoantibody result) was 2 months (range 1–53 months). The most common prodromal symptoms were fever or flu-like symptoms (10/30, 33.3%), presenting mostly in anti-NMDAR encephalitis (8/19, 42.1%). One anti-NMDAR patient had herpes simplex virus (HSV) encephalitis confirmed by positive PCR in the CSF, and NMDAR encephalitis developed about a month after discharge from the hospital. Four additional patients

TABLE 1 | Demographic data and tested sample types in AE patients.

	Autoimmune encephalitis ($n = 30$)	anti-NMDAR ($n = 19$)	anti-LGI1 ($n = 6$)	anti-GABABR ($n = 3$)	anti-Caspr2 ($n = 3$)
Age (range)	39.3 (1-75)	32.5 (1-75)	46.8 (3-65)	47 (16-67)	47.7 (3–72)
Sex (M/F)	19:11	11:8	5:1	2:1	2:1
Cured Complete recovery (n, %)*	20 (66.7%)	14 (73.7%)	4 (66.7%)	0	3 (100%)
Death (n, %)	3 (10%)	1 (5.3%)	1 (16.7%)	1 (33.3%)	0
Relapse (n, %)	1 (3.3%)	1 (5.3%)	0	0	0
Tested sample types					
CSF-serum pairs (n, %)	20 (66.7%)	12 (63.2%)	4 (66.7%)	3 (100%)	2 (66.7%)
Only CSF (n, %)	2 (6.7%)	2 (10.5%)	-	-	-
Only sera (n, %)	8 (26.7%)	5 (26.3%)	2 (33.3%)	-	1 (33.3%)

**Complete recovery was defined as the proportion of AE patients with mRS score of 0 at the last visit following treatment (median: 33 months; range: 1–77).*

M, male; F, female; NMDAR, N-methyl-D-aspartate receptor; LGI1, leucine-rich, glioma inactivated 1; GABABR, γ-aminobutyric acid receptor-B; Caspr2, contactin-associated protein-like 2, CSF, cerebrospinal fluid.

with anti-NMDAR encephalitis had HSV IgM in the serum (**Supplementary Table 1**).

The most common initial presentations were psychiatric symptoms before the onset of neurologic dysfunction (17/30, 56.7%) (**Supplementary Table 2**): 11 patients with anti-NMDAR, 3 patients with LGI1, 2 patients with GABABR and 2 patients with Caspr2. During the disease course, psychiatric symptoms were altogether present in 25/30 (83.3%) of AE patients. In our cohort, psychiatric symptoms included the presence of one or more of the following: anxiety, apathy, aggressiveness, agitation, depression, hypersexuality, disorientation, visual/auditory hallucinations, behavioral changes, cognitive deficit, psychomotor retardation/agitation, catatonia, consciousness disorder, and mutism. The most common psychiatric symptom was disorientation (14/30), followed by visual/auditory hallucinations (7/30), psychomotor retardation (7/30) and behavioral changes (6/30) (**Figure 1A**). In pediatric cases (age $<$ 10 years: Patient 13, 14, 18, and 30), psychiatric symptoms included the presence of one or more of the following: disorientation, behavioral changes and catatonia.

Other clinical features included seizures (22/30), memory loss (15/30), insomnia (7/30), speech disorders such as dysarthria or aphasia (5/30), status epilepticus (4/30) and ataxia (3/30). Piloerection, cerebellar symptoms, neuropathy and skin rashes occurred in singular cases, respectively. Involuntary movements, such as dyskinesia, dystonia or choreoathetosis were the most common in NMDAR encephalitis (9/19). Orofacial dyskinesia was present in 6/19 anti-NMDAR positive patients accompanied with hand dyskinesia in two patients. Dystonia occurred in two patients with NMDAR encephalitis, and choreoathetosis was observed in one NMDAR patient. Dysautonomia was present in one anti-NMDAR positive patient. Hyponatraemia (5/6) and faciobrachial dystonic seizures (3/6) were the most frequent in the LGI1 patient group. Clinical features of the different types of AE are summarized in **Figure 1B**.

Regarding associated tumors, eight cases (26.7%) were observed: in three anti-NMDAR, three anti-GABABR, one anti-LGI1 and one anti-Caspr2 patients. Five patients were male (62.5%) and median age was 62.5 years (range: 16–72 years). The most common tumor type was lung carcinoma (large cell lung carcinoma in one NMDAR patient and small cell lung carcinoma in two GABABR patient). In most cases (5/8, 62.5%), positive neuronal autoantibody test result preceded the detection of associated tumor. Median time from autoantibody positivity until detection of tumor was 15 months (**Supplementary Table 3**).

Auxiliary Examinations of AE Patients

CSF was analyzed in 80% (24/30) of AE patients (**Figure 2A**). Abnormal CSF findings, such as pleocytosis (white blood cell count $<$ 5 cells/mm^3), the presence of oligoclonal bands (OCB), increased protein level ($>$ 450 mg/L) and/or elevated IgG index ($>$ 0.65) were observed in 13/24 of the tested AE patients. The majority of patients with anti-NMDAR (8/15) and anti-GABABR (2/3) had abnormal CSF results. Pleocytosis (2/15, 13.3%) and OCB (5/15, 33.3%) were detected exclusively in NMDAR patients. Elevated IgG index was found in one anti-NMDAR and one anti-GABABR patient. Altogether, increased protein level was the most common abnormal CSF finding (9/24, 37.5%), accounting for all abnormal CSF cases in both the LGI1 and Caspr2 patient groups (**Figure 2A**).

We were able to evaluate EEG data in 29 patients. EEG abnormalities were observed in 14/29 patients (**Supplementary Table 4**). The most common abnormal EEG findings were focal slowing (6/30) and interictal epileptiform discharges (4/30) (**Supplementary Figure 1**).

Brain MRI examination was evaluated in 27 patients. Abnormalities on brain MRI were observed in 14/27 patients (**Supplementary Table 5**). Abnormal brain MRI results were mainly unilateral or bilateral lesions in the insula/hippocampus (13/30) (**Figures 2B,C**). In 7/14 (50%) of AE patients with abnormal brain MRI results, follow-up brain MRI examinations were conducted (median: 24 months, range: 2–37 months), which revealed hippocampal sclerosis or cerebral atrophy in three anti-LGI1 and two anti-NMDAR positive patients (5/7, 71.4% of patients with follow-up brain MRI result).

Treatment and Outcomes

In 24/30 of AE patients, first-line immunotherapy was applied. Most commonly methylprednisolone pulse was used in combination with plasmapheresis (12/30). Other therapy included only the use of methylprednisolone (4/30) or in combination with intravenous immunoglobulin (5/30). Combination of all three types of immunotherapy was administered in 4/30 cases: three anti-NMDAR patients (mRS 5 at admission) presenting with psychiatric symptoms, seizures and involuntary movements, and one anti-LGI1 patient (mRS 3 at admission) with FBDS, hyponatraemia, dysautonomia and insomnia required such combination. All four AE patients had good outcome at the last visit (mRS 0). In one case, plasmapheresis was applied alone. Most patients (22/30, 73.3%) responded to the first-line therapy. Second-line therapy was introduced with azathioprine or rituximab or with the combination of the two in four cases with anti-NMDAR encephalitis presenting with severe disability during admission (mRS $\geq$ 4) and with no significant improvement to first-line therapy (**Supplementary Table 6**). Azathioprin and/or rituximab was continued as maintenance therapy in three out of these four AE patients, due to residual symptoms (memory loss, psychiatric symptoms). Antiepileptics were used exclusively in 6/30 (20%) of AE patients, presenting predominantly with seizures and showing good outcome at the last visit (mRS median: 0; range: 0–1).

Introduction of immunotherapy was followed by at least one repeated neuronal cell surface autoantibody test in 20/30 (66.7%) of AE patients (median: 8.3 months, range: 1–47). Antibody positivity persisted in 11/20 (55%) of repeatedly tested patients (seven anti-NMDAR, three anti-GABABR and one anti-LGI1 encephalitis patients). The median hospital stay of AE patients was 23 days. Patients were severely impaired on admission, with a median mRS score of 4 (range 2–5). Most AE patients showed significant improvement after treatment and 25/30 (83.3%) achieved a good outcome (mRS $\leq$ 2). Median follow-up duration

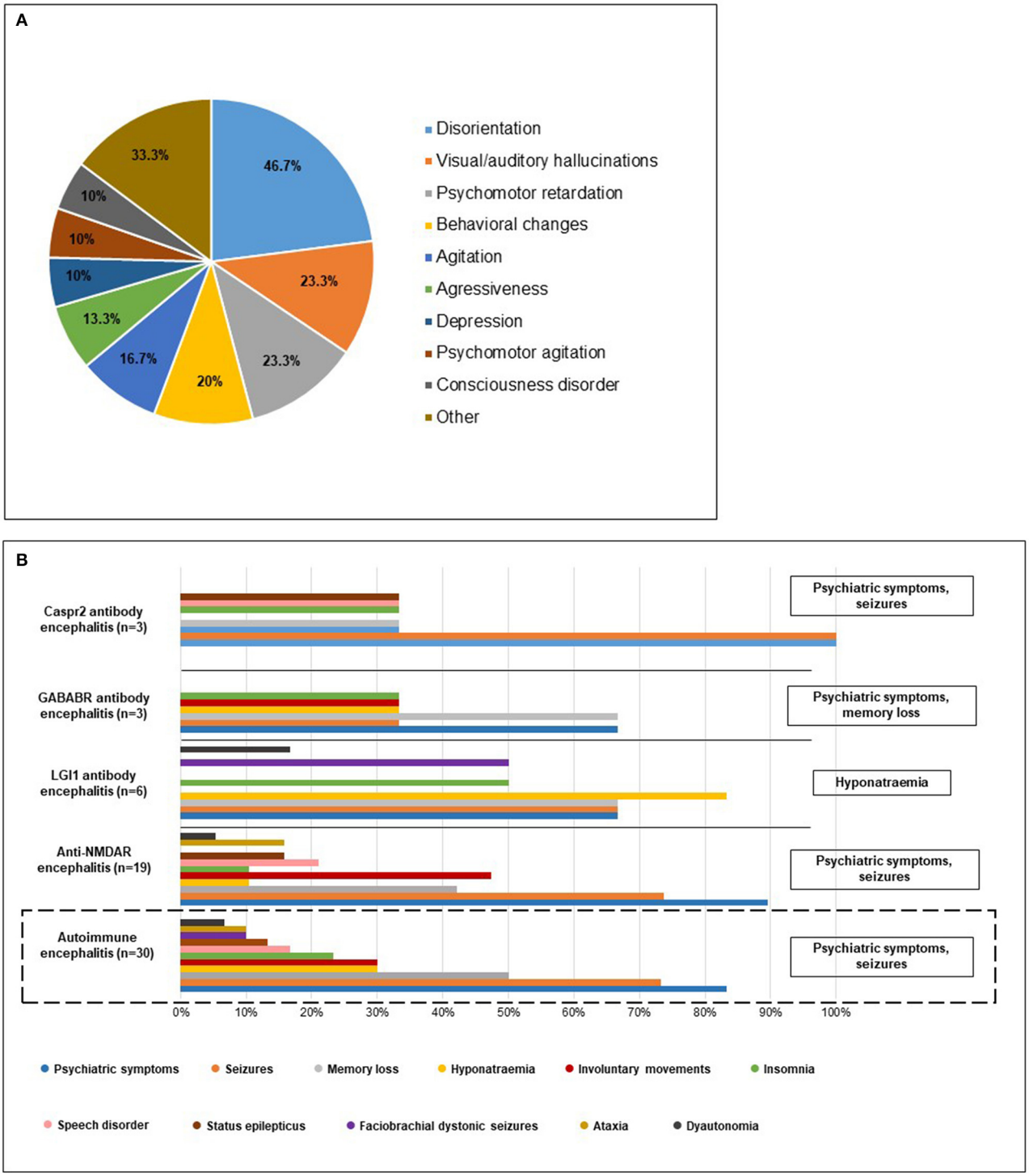


FIGURE 1 | (A) Psychiatric symptoms of AE patients. The diagram shows the distribution of psychiatric symptoms among the 30 AE patients in our cohort. Most common symptoms included disorientation, visual/auditory hallucinations, psychomotor retardation and behavioral changes. Frequency of rare psychiatric symptoms such as anxiety, cognitive deficit, catatonia, apathy, hypersexuality, and mutism are included in "Other." **(B)** Characteristic clinical symptoms of AE patients. The bars show the frequency of different clinical features of AE patients. The diagram at the bottom shows that psychiatric symptoms and seizures were the most common in the 30 AE patients in our cohort. The other diagrams indicate distribution of clinical features separately in different AE types. Different distribution of clinical symptoms was observed in anti-LGI1 and anti-GABABR patients: in LGI1 encephalitis hyponatraemia and in GABABR encephalitis besides psychiatric symptoms memory loss was among the most common symptoms.

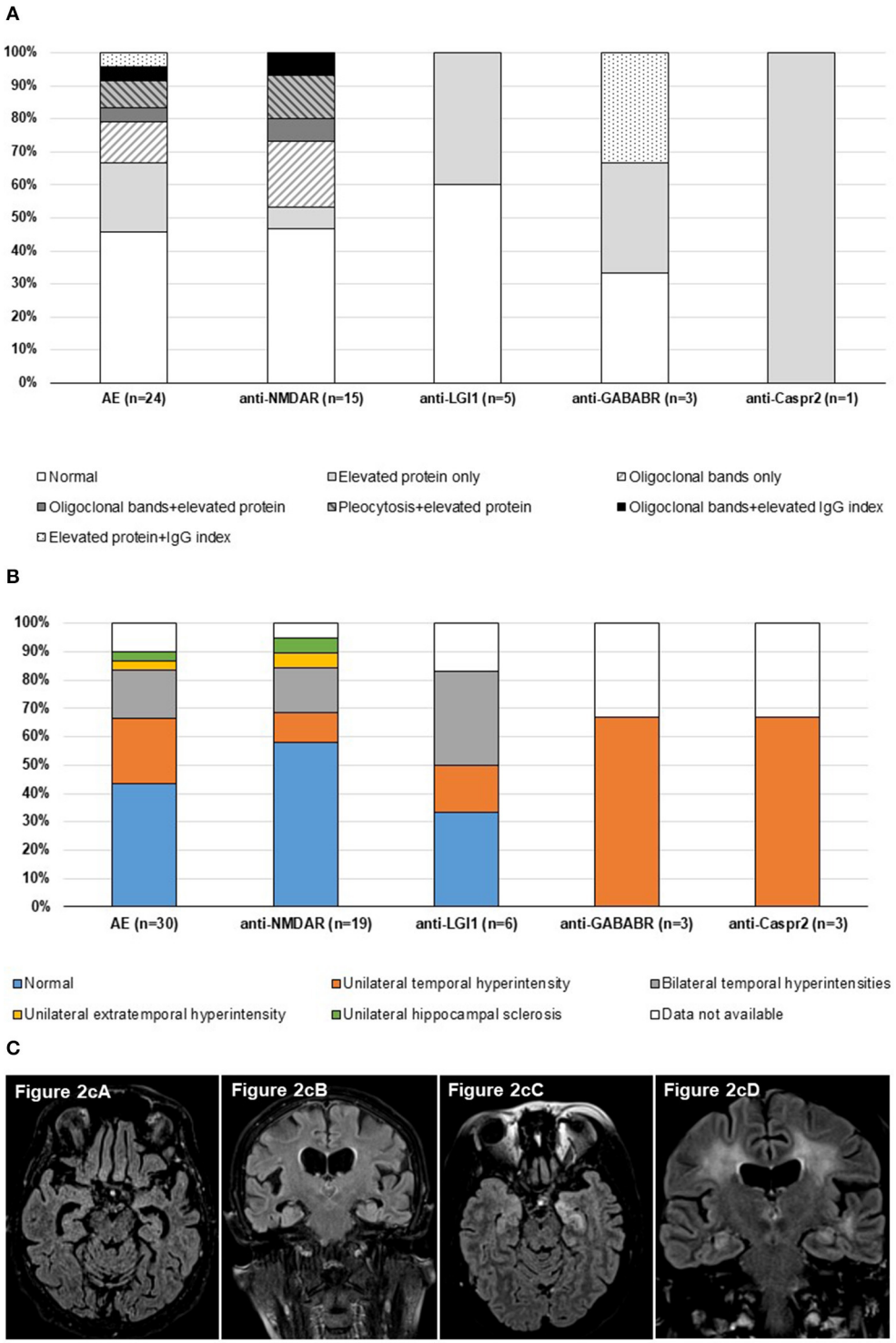


FIGURE 2 | (A) Composition of cerebrospinal fluid in AE patients. The bars demonstrate CSF analyses result of 24 AE patients from our cohort. At least one abnormal CSF finding was found in 13 AE patients, most commonly in anti-NMDAR and anti-GABABR patients. Pleocytosis and OCB were detected only in NMDAR encephalitis. **(B)** Brain MRI findings in AE patients. The diagram shows brain MRI result of 27 AE patients from our cohort. In 14 AE patients, abnormalities were found, mainly in the form of unilateral or bilateral temporal hyperintensities. **(C)** Axial and coronal FLAIR brain images from patient 8 (cA, cB) and patient 24 (cC, cD). Patient 8 was a 75-year-old female patient, who had anti-NMDAR autoantibody positivity detected in CSF and had hyperintensity and contrast enhancement within bilateral medial temporal lobes (cA-axial 3D FLAIR, **c**B-coronal T2 FLAIR). Patient 24 was a 50-year-old female patient, who had anti-LGI1 autoantibody positivity detected in serum and had bilateral limbic encephalitis, dominant in the left temporal lobe, both on axial (cC) and coronal T2 FLAIR images (cD).

was 33 months (range 1–77) (**Table 2**). Five patients had a mRS $\geq$ 3 at the last visit, including one patient with anti-NMDAR and one patient with anti-GABABR who died of consequences of associated lung tumor and one patient with anti-LGI1 who died of deep vein thrombosis. One patient with anti-GABABR and small cell lung cancer was bedridden and required continuous nursing. One patient with anti-NMDAR had persistent cognitive deficit and memory loss, requiring some help as being unable to perform all previous activities. In our cohort, AE patients with associated tumor ($n = 8$) had a significantly higher mRS score at the time of the last visit (median: 2.5, range: 0–6) compared to AE patients without tumor ($n = 22$; mRS at the last visit median: 0, range: 0–3) ($p = 0.045$).

The rate of complete recovery in AE patients (follow-up median: 33 months; range: 1–77 months), showed favorable prognosis (20/30), with the highest rate in the anti-Caspr2 patient group. Relapses were uncommon in AE patients (1/30): only one male patient relapsed, whose anti-NMDAR positivity persisted in CSF during repeated autoantibody tests (follow-up: 47 months; number of autoantibody tests: 4). Death occurred in 3/30 of AE patients (**Table 1**).

Comparison of AE Patients Based on Signs of CNS Inflammation and Associated Autoantibody Types

We analyzed the cohort based on the presence of CNS inflammatory markers, i.e. CSF inflammatory changes and brain MRI abnormalities. We defined CNS inflammation as the presence of at least one of the CSF inflammatory markers, such as pleocytosis (white blood cell count $>$ 5 cells/mm^3), oligoclonal bands, elevated protein or IgG index and/or brain MRI lesions suggestive of encephalitis (mesial temporal T2 signal hyperintensity or signs of demyelination). CNS inflammation was present in 19/30 patients (63.3%): both CSF and MRI abnormalities were detected in 7/30 patients (23.3%), 6/30 patients (20%) had brain MRI lesions suggestive of encephalitis without altered CSF findings, and 6/30 patients (20%) had signs of inflammation in CSF, but no abnormal brain MRI findings. In the remaining 11/30 cases, no changes indicating CNS inflammation were detected. **Table 2** shows a comparison of clinical data of AE patients presenting with or without CNS inflammatory markers. In the patient group with inflammatory changes, age of onset was significantly higher ($p = 0.024$). No significant differences in sex, frequency of tumor association, time to diagnosis, prognosis and type of immunotherapy were observed between AE patients with and without CNS inflammatory markers.

We also analyzed AE patients based on the type of neuronal autoantibodies (**Table 2**). Patients were classified as anti-NMDAR positive and positive for other AE-related antibodies (anti-LGI1, anti-GABABR, anti-Caspr2). NMDAR encephalitis patients ($n = 19$) were in more severe condition at the onset of the disease with significantly higher mRS score at the time of diagnosis (median: 5, range: 2-5) compared to LGI1, GABABR and Caspr2 encephalitis ($n = 11$; mRS score at the diagnosis median: 3, range: 2–5) ($p = 0.028$). A trend of longer time to diagnosis was observed in the non-NMDAR patient group compared to NMDAR positive patients ($p = 0.063$). However, in two LGI1-encephalitis cases, final diagnosis of AE was retrospectively confirmed after the introduction of cell-based assay in our laboratory in 2012. No significant differences were found in sex, frequency of tumor association, prognosis and type of immunotherapy between anti-NMDAR positive AE patients and patients positive for other AE-related antibodies (anti-LGI1, anti-GABABR, anti-Caspr2).

DISCUSSION

We aimed to examine the characteristics of AE including clinical, laboratory, MRI features and outcome of AE in a Hungarian cohort. In accordance with previous publications of other series (6, 16–18), the most common autoimmune encephalitis type was anti-NMDAR encephalitis, followed by anti-LGI1 encephalitis (3, 19, 20). The study by Bien et al. (21) reported 576 antibody-positive patients during testing of 10,919 patients for a broad panel of neural antibodies, including onconeural and neuronal cell surface autoantibodies. Our previous study (10) included results of 60 neuronal cell surface autoantibody positive patients (anti-NMDAR, anti-LGI1, anti-GABABR, anti-Caspr2, anti-AMPAR1, 2) and our recent study exclusively included both serologically (neuronal cell surface antibody positive, but not onconeural autoantibody positive) and clinically positive definite AE patients. The distinct results in autoantibody frequencies detected in the two laboratories, may be due to the exclusive inclusion of neuronal cell surface autoantibody positive AE patients in our study, making it impossible to compare accurately the data of autoantibody frequencies. In our cohort, the sex ratio was close to equal, with NMDAR encephalitis occurring slightly more frequently in men than women. Similar sex ratio was observed in a cohort of Chinese AE patients (12), although, published data claims that anti-NMDAR encephalitis affects predominantly young women (median age, 21 years) (3, 22). Dalmau et al. (3) reported male predominance in LGI1, GABABR and Caspr2 encephalitis, which was also observed in our cohort, although, the median age of our AE patients were 45–50 years compared to median values of 60–65 years in previous publications (3, 23).

In 70–86% of anti-NMDAR encephalitis patients, prodromal symptoms, such as headache, fever, nausea and vomiting were present (19, 24, 25). In our cohort, HSV infection occurred in one patient, who had secondary NMDAR encephalitis one month after HSV encephalitis. Previous publications (26) have reported HSV infection as being the most common viral trigger in AE patients and a possible trigger of anti-NMDAR encephalitis. The precise mechanism of HSV triggered AE is not clearly defined. Molecular mimicry or breakdown of immunological tolerance may be in the background (27). It has been stated that the most common symptoms of AE are psychiatric symptoms, seizures, involuntary movements, memory loss and sleep disorders (23). Besides these characteristic features, some rare manifestations, such as piloerection also emphasized in a systematic review and related to LGI1 encephalitis (28), cerebellar

TABLE 2 | Comparison of AE patients regarding the presence of CNS inflammation and associated autoantibody type.

Variables	With CNS inflammation ($n = 19$)	Without CNS inflammation ($n = 11$)	anti-NMDAR encephalitis ($n = 19$)	anti-LGI1, anti-GABABR, anti-Caspr2 encephalitis ($n = 11$)
Age (range)	54 (16-75)	17 (1-70)	32.5 (1-75)	58 (3-72)
Male (*n*, %)	14 (73.7%)	5 (45.5%)	11 (57.9%)	8 (72.7%)
Tumor (*n*, %)	6 (31.6%)	2 (18.9%)	3 (15.8%)	5 (45.5%)
Diagnostic delay (median, months)	3	1	1	5
mRS at diagnosis (median)	3	5	5	3
mRS at last visit (median)	0	0	0	0
Status at Last Visit				
mRS 0–2 (*n*, %)	15 (78.9%)	10 (90.9%)	17 (89.5%)	8 (72.7%)
mRS 3-6 (*n*, %)	4 (21.1%)	1 (9.1%)	2 (10.5%)	3 (27.3%)
Immunotherapy				
Only steroid (*n*, %)	3 (15.8%)	1 (9.1%)	3 (15.8%)	1 (9.1%)
Steroid+IVIG (*n*, %)	0	2 (18.9%)	1 (5.3%)	1 (9.1%)
Steroid+PE (*n*, %)	10 (52.6%)	2 (18.9%)	5 26.3%)	5 (45.5%)
Steroid+IVIG+PE (*n*, %)	1 (5.3%)	3 (27.3%)	1 (5.3%)	1 (9.1%)
Second-line therapy (*n*, %)	2 (10.5%)	2 (18.9%)	4 (21.1%)	0

CNS inflammation was defined as the presence of at least one of the CSF inflammatory markers, such as pleocytosis (white blood cell count <5cells/mm3), oligoclonal bands, elevated protein or IgG index and/or brain MRI lesions suggestive of encephalitis (mesial temporal T2 hypersignal or signs of demyelination).
CNS, central nervous system; NMDAR, N-methyl-D-aspartate receptor; LGI1, leucine-rich, glioma inactivated 1; GABABR, γ-aminobutyric acid receptor-B; Caspr2, contactin-associated protein-like 2; mRS, modified Rankin scale score; IVIG, intravenous immunoglobulin; PE, plasmapheresis.

symptoms, neuropathy and skin rashes were also observed in our cohort. Previous publications have reported of FBDS as a typical symptom of anti-LGI1 encephalitis, often occurring a few weeks before onset of cognitive deficit in 26–71% of patients (20). In a retrospective study in the UK (29), 77% (20/26) of anti-LGI1 encephalitis patients experienced FBDS prior to the development of limbic encephalitis and found that early immunotherapy for FBDS might prevent progression to cognitive impairment. A prospective study of nine patients with anti-LGI1 encephalitis also revealed the beneficial effect of early immunotherapy (30). In our cohort of AE patients, FBDS was exclusively present in the LGI1 encephalitis patients with a lower prevalence (50%) compared to previous publications. Besides FBDS, hyponatraemia was also predominant in this group.

Examination of CSF has an important role in diagnosis, as the presence of pleocytosis is included in the diagnostic criteria for AE (7), although AE associated with autoantibodies against LGI1 and Caspr2 sometimes lack signs of inflammation in the CSF. The study of Hébert et al. (31) reported prevalence of CSF inflammatory markers, including elevated white blood cell count, elevated protein concentration and OCB in 95 patients with early active AE. Similarly to our study, where 46% of AE patients had normal CSF results upon testing, in the cohort of Hébert et al. 44% of AE patients lacked CSF pleocytosis, 27% of patients, in addition to the lack of CSF pleocytosis, had normal protein concentration in the CSF and 14% of AE patients, besides the mentioned two CSF parameters, lacked OCB. In a retrospective study of anti-LGI1 encephalitis patients (32), CSF pleocytosis was identified in 23% of patients. Meanwhile, NMDAR and GABABR encephalitis are frequently associated with CSF inflammatory changes, such as pleocytosis and/or the presence of OCB (33–35). In a cohort of 100 patients with anti-NMDAR encephalitis (24) abnormal CSF findings were described in 95% of patients, including CSF pleocytosis in 91%, increased total protein levels in 32% and OCB in 66.7% of anti-NMDAR patients. In a study analyzing clinical data of 44 anti-NMDAR encephalitis patients (36), reported CSF pleocytosis in 68% of patients, OCB was present in altogether 52% of patients during disease course. These findings were also observed in our study, where in anti-LGI1 and anti-Caspr2 positive patients, mainly normal CSF results were found, or increased total protein levels were detected in a few cases. In contrast with previous publications (35), CSF pleocytosis and OCB were exclusively detected in NMDAR patients. Other abnormal CSF findings, such as elevated IgG index and/or increased total protein levels were detected in the GABABR patient group. Data of prevalence of tumor association was also confirmed in our study, where 75% of AE patients presenting with tumor were anti-NMDAR and anti-GABABR positive. Ovarian teratoma is considered the most frequent tumor in anti-NMDAR encephalitis (6), but no association was found in our cohort. In a Chinese series, it was also rare and was only found in 2/72 patients (12). In GABABR encephalitis, the dominant tumor type was small cell lung cancer, occurring in 66.7% of anti-GABABR positive patients, which is in agreement with the findings of Hermetter et al. (6).

Most AE patients in our study showed favorable prognosis. In our cohort, the rate of complete recovery (66.7%) was slightly lower and death rate (10%) was mildly higher compared to data published by Deng et al. (complete recovery: 81.3%, death rate: 6%) (12). In our cohort, relapses occurred exclusively among anti-NMDAR patients (1/19, 5.3% of anti-NMDAR patients). In a cohort of Chinese patients, relapses were also uncommon,

occurring in 7/86 (8.1%) AE patients, mainly affecting NMDAR patients (5/72, 6.9% of anti-NMDAR patients) (12). However, in a cohort of Argentine AE patients 25% of anti-NMDAR patients had relapses, but relapses also occurred in 25% of anti-LGI1 patients (15). Majority of the patients received first-line immunotherapy, steroid solely, or in combination with intravenous immunoglobulin or plasmapheresis, which had a good curative effect in most AE patients.

In 63.3% of AE patients in our cohort, signs of inflammation were detected in CSF and/or brain MRI, but no significant correlation was found between inflammatory markers and prognosis. In the study of Escudero et al. (8) a retrospective clinical analysis was conducted in 155 neuronal cell surface autoantibody positive patients with $\geq$ 60 years of age, but no brain MRI and CSF inflammatory changes were observed. In the cohort of Escudero et al. the most common autoantibody type was anti-LGI1 and the frequency of patients without evidence of CNS inflammation ranged from 25% (LGI1 antibodies) to 7% (GABABR antibodies). Escudero et al. reported higher frequency of non-inflammatory profile in anti-LGI1 patients $\geq$ 60 years of age (25%), compared to younger patients (age $<$ 60 years; 3%). In our study we also reported an overall significantly higher age of onset in AE patients presenting with inflammatory changes. Direct neuronal dysfunction caused by autoantibodies besides inflammatory infiltrates and blood–brain barrier abnormalities may explain this observation (37). Previous publications have found association of CSF changes with worse outcome (38, 39), although early CSF and brain MRI abnormalities did not show a strong correlation with disease outcome (40).

In conclusion, characteristics of AE in our Hungarian multicenter retrospective study are in agreement with previous findings. Anti-NMDAR patients presented with more severe disability at admission compared to anti-LGI1, anti-GABABR and anti-Caspr2 encephalitis. Presence of tumor was associated with worse outcome in AE patients compared to those patients without cancer. However, none of the anti-NMDAR encephalitis female patients had ovarian teratoma. Thirty seven percent of patients lacked presence of both CSF inflammatory markers and brain MRI abnormalities. This observation, in addition to the role of auxiliary examinations (CSF analysis, EEG, brain MRI), emphasizes the importance of clinical presentation and autoantibody testing in diagnostic workflow. Our findings also highlight the significance of early introduction of first-line immunotherapy that resulted in favorable outcome in most AE patients in our cohort.

Our study is limited due to the retrospective data collection performed by clinicians using an online questionnaire, which may result in inadequate accuracy during reporting. The study design precludes the ability to address characteristics of AE that were not directly questioned or consistently recognized (for example, among sleep dysfunctions exclusively the data regarding the presence of insomnia was collected). In our study, due to the low number of pediatric cases with age $<$ 10 years (four cases), we could not confidently determine characteristics of pediatric patients. Although, the study has modest sample size, it summarizes detailed clinical data of 35 neuronal surface antibody positive patients from the 60 patients with positive autoantibody test results (N_{total} = 1,034 patients with suspected AE) from 2012 through 2018 in Hungary published in our previous study (10). Our data confirms results of previous publications and further clarifies clinical data of AE patients with neuronal cell surface autoantibodies.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical Research Council of Hungary (number of approval: 49709-2/2019/EKU). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZH designed and conceptualized the study, had a major role in the acquisition of data, analyzed the data, and wrote the first draft of the manuscript. BB designed and conceptualized the study and reviewed the manuscript. DS executed the statistical analysis and reviewed the manuscript. GO, MS, FN, TC, ZM, JN, and CR had a major role in acquisition of data and revised the manuscript. ZI and TB designed and conceptualized the study, analyzed the data and revised the manuscript for intellectual content.

FUNDING

This study was supported by GINOP-232-15-2016-00050 and EFOP 361-16-2016-00004 grants. The research was financed by the Thematic Excellence Program 2020—Institutional Excellence Sub-programme of the Ministry for Innovation and Technology in Hungary, within the framework of the second thematic programme of the University of Pécs.

ACKNOWLEDGMENTS

The authors thank Szabolcs Fekete (Department of Immunology and Biotechnology, Clinical Center, University of Pécs Medical School, Pécs, Hungary) for his technical support in creating the online questionnaire for data collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.611597/full#supplementary-material

REFERENCES

1. Dubey D, Pittock SJ, Kelly CR, McKeon A, Lopez-Chiriboga AS, Lennon VA, et al. Autoimmune encephalitis epidemiology and a comparison to infectious encephalitis. *Ann Neurol.* (2018) 83:166–77. doi: 10.1002/ana.25131
2. Armangue T, Leypoldt F, Dalmau J. Autoimmune encephalitis as differential diagnosis of infectious encephalitis. *Curr Opin Neurol.* (2014) 27:361–8. doi: 10.1097/WCO.0000000000000087
3. Dalmau J, Graus F. Antibody-Mediated Encephalitis. *N Engl J Med.* (2018) 378:840–51. doi: 10.1056/NEJMra1708712
4. Leypoldt F, Armangue T, Dalmau J. Autoimmune encephalopathies. *Ann N Y Acad Sci.* (2015) 1338:94–114. doi: 10.1111/nyas.12553
5. Dutra LA, Abrantes F, Toso FF, Pedroso JL, Barsottini OGP, Hoftberger R. Autoimmune encephalitis: a review of diagnosis and treatment. *Arq Neuropsiquiatr.* (2018) 76:41–9. doi: 10.1590/0004-282X20170176
6. Hermetter C, Fazekas F, Hochmeister S. Systematic Review: Syndromes, Early Diagnosis, and Treatment in Autoimmune Encephalitis. *Front Neurol.* (2018) 9:706. doi: 10.3389/fneur.2018.00706
7. Graus F, Titulaer MJ, Balu R, Benseler S, Bien CG, Cellucci T, et al. A clinical approach to diagnosis of autoimmune encephalitis. *Lancet Neurol.* (2016) 15:391–404. doi: 10.1016/S1474-4422(15)00401-9
8. Escudero D, Guasp M, Ariño H, Gaig C, Martínez-Hernández E, Dalmau J, et al. Antibody-associated CNS syndromes without signs of inflammation in the elderly. *Neurology.* (2017) 89:1471–5. doi: 10.1212/WNL.0000000000004541
9. Wagner JN, Kalev O, Sonnberger M, Krehan I, von Oertzen TJ. Evaluation of Clinical and Paraclinical Findings for the Differential Diagnosis of Autoimmune and Infectious Encephalitis. *Front Neurol.* (2018) 9:434. doi: 10.3389/fneur.2018.00434
10. Hayden Z, Böröcz K, Csizmadia Z, Balogh P, Kellermayer Z, Bodó K, et al. Single-center study of autoimmune encephalitis-related autoantibody testing in Hungary. *Brain Behav.* (2019) 9:e01454. doi: 10.1002/brb3.1454
11. Lim JA, Lee ST, Moon J, Jun JS, Kim TJ, Shin YW, et al. Development of the clinical assessment scale in autoimmune encephalitis. *Ann Neurol.* (2019) 85:352–8. doi: 10.1002/ana.25421
12. Deng S, Qiu K, Liu H, Wu X, Lei Q, Lu W. Clinical Characteristics and Short-Term Prognosis of Autoimmune Encephalitis: A Single-Center Cohort Study in Changsha, China. *Front Neurol.* (2019) 10:539. doi: 10.3389/fneur.2019.00539
13. Nóbrega PR, Pitombeira MS, Mendes LS, Krueger MB, Santos CF, Morais NMM, et al. Clinical Features and Inflammatory Markers in Autoimmune Encephalitis Associated With Antibodies Against Neuronal Surface in Brazilian Patients. *Front Neurol.* (2019) 10:472. doi: 10.3389/fneur.2019.00472
14. Szots M, Blaabjerg M, Orsi G, Iversen P, Kondziella D, Madsen CG, et al. Global brain atrophy and metabolic dysfunction in LGI1 encephalitis: a prospective multimodal MRI study. *J Neurol Sci.* (2017) 376:159–65. doi: 10.1016/j.jns.2017.03.020
15. Melamud LI, Fernández VC, Manin A, Villa AM. Autoimmune encephalitis and immune therapy: lessons from Argentina. *Acta Neurol Belg.* (2020) 120:565–72. doi: 10.1007/s13760-018-1013-x
16. Dalmau J, Geis C, Graus F. Autoantibodies to Synaptic Receptors and Neuronal Cell Surface Proteins in Autoimmune Diseases of the Central Nervous System. *Physiol Rev.* (2017) 97:839–87. doi: 10.1152/physrev.00010.2016
17. Gable MS, Sheriff H, Dalmau J, Tilley DH, Glaser CA. The frequency of autoimmune N-methyl-D-aspartate receptor encephalitis surpasses that of individual viral etiologies in young individuals enrolled in the California Encephalitis Project. *Clin Infect Dis.* (2012) 54:899–904. doi: 10.1093/cid/cir1038
18. Granerod J, Ambrose HE, Davies NW, Clewley JP, Walsh AL, Morgan D, et al. Causes of encephalitis and differences in their clinical presentations in England: a multicentre, population-based prospective study. *Lancet Infect Dis.* (2010) 10:835–44. doi: 10.1016/S1473-3099(10)70222-X
19. Dalmau J, Lancaster E, Martinez-Hernandez E, Rosenfeld MR, Balice-Gordon R. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. *Lancet Neurol.* (2011) 10:63–74. doi: 10.1016/S1474-4422(10)70253-2
20. van Sonderen A, Petit-Pedrol M, Dalmau J, Titulaer MJ. The value of LGI1, Caspr2 and voltage-gated potassium channel antibodies in encephalitis. *Nat Rev Neurol.* (2017) 13:290–301. doi: 10.1038/nrneurol.2017.43
21. Bien CG, Bien CI, Dogan Onugoren M, De Simoni D, Eigler V, Haensch CA, et al. Routine diagnostics for neural antibodies, clinical correlates, treatment and functional outcome. *J Neurol.* (2020) 267:2101–14. doi: 10.1007/s00415-020-09814-3
22. Hébert J, Riche B, Vogrig A, Muñiz-Castrillo S, Joubert B, Picard G, et al. Epidemiology of paraneoplastic neurologic syndromes and autoimmune encephalitides in France. *Neurol Neuroimmunol Neuroinflamm.* (2020) 7. doi: 10.1212/NXI.0000000000000883
23. Newman MP, Blum S, Wong RC, Scott JG, Prain K, Wilson RJ, et al. Autoimmune encephalitis. *Intern Med J.* (2016) 46:148–57. doi: 10.1111/imj.12974
24. Dalmau J, Gleichman AJ, Hughes EG, Rossi JE, Peng X, Lai M, et al. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. *Lancet Neurol.* (2008) 7:1091–8. doi: 10.1016/S1474-4422(08)70224-2
25. Ma C, Wang C, Zhang Q, Lian Y. Emerging role of prodromal headache in patients with anti-N-methyl-D-aspartate receptor encephalitis. *J Pain Res.* (2019) 12:519–26. doi: 10.2147/JPR.S189301
26. Armangue T, Spatola M, Vlagea A, Mattozzi S, Cárceles-Cordon M, Martinez-Heras E, et al. Frequency, symptoms, risk factors, and outcomes of autoimmune encephalitis after herpes simplex encephalitis: a prospective observational study and retrospective analysis. *Lancet Neurol.* (2018) 17:760–72. doi: 10.1016/S1474-4422(18)30244-8
27. Leypoldt F, Titulaer MJ, Aguilar E, Walther J, Bönstrup M, Havemeister S, et al. Herpes simplex virus-1 encephalitis can trigger anti-NMDA receptor encephalitis: case report. *Neurology.* (2013) 81:1637–9. doi: 10.1212/WNL.0b013e3182a9f531
28. Tényi D, Bóné B, Horváth R, Komoly S, Illés Z, Beier CP, et al. Ictal piloerection is associated with high-grade glioma and autoimmune encephalitis-Results from a systematic review. *Seizure.* (2019) 64:1–5. doi: 10.1016/j.seizure.2018.11.009
29. Irani SR, Michell AW, Lang B, Pettingill P, Waters P, Johnson MR, et al. Faciobrachial dystonic seizures precede Lgi1 antibody limbic encephalitis. *Ann Neurol.* (2011) 69:892–900. doi: 10.1002/ana.22307
30. Irani SR, Stagg CJ, Schott JM, Rosenthal CR, Schneider SA, Pettingill P, et al. Faciobrachial dystonic seizures: the influence of immunotherapy on seizure control and prevention of cognitive impairment in a broadening phenotype. *Brain.* (2013) 136(Pt 10):3151–62. doi: 10.1093/brain/awt212
31. Hébert J, Gros P, Lapointe S, Amtashar FS, Steriade C, Maurice C, et al. Searching for autoimmune encephalitis: Beware of normal CSF. J Neuroimmunol. (2020) 345:577285. doi: 10.1016/j.jneuroim.2020.577285
32. Ariño H, Armangué T, Petit-Pedrol M, Sabater L, Martinez-Hernandez E, Hara M, et al. Anti-LGI1-associated cognitive impairment: presentation and long-term outcome. *Neurology.* (2016) 87:759–65. doi: 10.1212/WNL.0000000000003009
33. van Sonderen A, Thijs RD, Coenders EC, Jiskoot LC, Sanchez E, de Bruijn MA, et al. Anti-LGI1 encephalitis: clinical syndrome and long-term follow-up. *Neurology.* (2016) 87:1449–56. doi: 10.1212/WNL.0000000000003173
34. van Sonderen A, Ariño H, Petit-Pedrol M, Leypoldt F, Körtvélyessy P, Wandinger KP, et al. The clinical spectrum of Caspr2 antibody-associated disease. *Neurology.* (2016) 87:521–8. doi: 10.1212/WNL.0000000000002917
35. Blinder T, Lewerenz J. Cerebrospinal Fluid Findings in Patients With Autoimmune Encephalitis-A Systematic Analysis. *Front Neurol.* (2019) 10:804. doi: 10.3389/fneur.2019.00804
36. Irani SR, Bera K, Waters P, Zuliani L, Maxwell S, Zandi MS, et al. N-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorder of both sexes. *Brain.* (2010) 133(Pt 6):1655–67. doi: 10.1093/brain/awq113
37. Lancaster E, Dalmau J. Neuronal autoantigens–pathogenesis, associated disorders and antibody testing. Nat Rev Neurol. (2012) 8:380–90. doi: 10.1038/nrneurol.2012.99

38. de Montmollin E, Demeret S, Brulé N, Conrad M, Dailler F, Lerolle N, et al. Anti-N-Methyl-d-Aspartate Receptor Encephalitis in Adult Patients Requiring Intensive Care. *Am J Respir Crit Care Med*. (2017) 195:491–9. doi: 10.1164/rccm.201603-0507OC
39. Wang W, Li JM, Hu FY, Wang R, Hong Z, He L, et al. Anti-NMDA receptor encephalitis: clinical characteristics, predictors of outcome and the knowledge gap in southwest China. *Eur J Neurol.* (2016) 23:621–9. doi: 10.1111/ene.12911
40. Broadley J, Seneviratne U, Beech P, Buzzard K, Butzkueven H, O'Brien T, et al. Prognosticating autoimmune encephalitis: a systematic review. *J Autoimmun.* (2019) 96:24–34. doi: 10.1016/j.jaut.2018.10.014

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Fwd: 9953317: Article Processing Charges

Beáta Bóné <bonebea@gmail.com>

2021. 05. 03., H, 9:43

Címzett: Dr. Hayden Zsófia <hayden.zsofia@pte.hu>

---------- Forwarded message ---------
Feladó: **Hindawi Invoicing** <invoices@hindawi.com>
Date: 2021. ápr. 29., Cs, 18:10
Subject: 9953317: Article Processing Charges
To: <bonebea@gmail.com>

Hindawi

Dear Dr. Beáta Bóné,

Thank you for choosing Hindawi to publish your manuscript

We are pleased to inform you that your manuscript, Toll-like receptor homolog CD180 expression is diminished on natural autoantibody producing B cells of patients with autoimmune CNS disorders, has been accepted for publication in the journal, Journal of Immunology Research. We will be in touch shortly to request electronic files for your manuscript.

The publication process will begin upon the receipt of these files.

As an open access journal, Journal of Immunology Research has an associated Article Processing Charge. The total charges for your manuscript 9953317, before any taxes, are USD 2100.

VAT charges will be applied for individuals resident in the UK and for institutions registered in the UK. VAT is calculated at the applicable rate, currently 20%, on the net USD amount and this VAT charge will be available for review on the invoice prior to confirmation.

What to do next?

This invoice is payable upon receipt. You can view the invoice for your article, confirm billing details, apply coupons and make payment through the following URL:

INVOICE DETAILS

You do not need to log in to your account to access the link. After entering your billing address information, you will be able to pay by credit card, PayPal or bank transfer. We are unable to accept payment by check.

If paying by **credit card**, we accept **Visa**, **Mastercard**, **Discover** and **Maestro**. If your credit card is not one of these then you may be able to use **PayPal** to make payment using your credit card.

If paying by **bank transfer**, please use invoice number in the payment reference and return a scanned copy of the bank payment authorization by email to invoices@hindawi.com to help us track your payment. Please note that bank transfer payments can take up to a week to arrive and will be confirmed as soon as funds have cleared.

If the payment needs to be made by your institution or by another author, you can forward them the invoice link in this email and they will be able to make payment directly. However, please note that as the author that submitted the article remains responsible for ensuring payment is made in full, you may be contacted if this institution or author does not make payment.

Got a question?

If you have any questions related to the invoice, just reply to this email and our Customer Service team will be happy to help.

Multiple Sclerosis and Related Disorders 51 (2021) 102923

Contents lists available at ScienceDirect

Multiple Sclerosis and Related Disorders

journal homepage: www.elsevier.com/locate/msard

Original article

Microstructural and functional brain abnormalities in multiple sclerosis predicted by osteopontin and neurofilament light

Gergely Orsi [a,b], Tamas Cseh [b], Zsofia Hayden [c], Gabor Perlaki [a,b], Szilvia Anett Nagy [a,b], Omar Giyab [d], Dorte Aalund Olsen [e], Jonna Skov Madsen [e,f], Timea Berki [c], Zsolt Illes [b,g,h,*]

[a] MTA-PTE Clinical Neuroscience MR Research Group, Eötvös Loránd Research Network (ELKH), Pecs, Hungary
[b] Department of Neurology, Medical School, University of Pecs, Pecs, Hungary
[c] Department of Immunology and Biotechnology, Medical School, University of Pecs, Pecs, Hungary
[d] Department of Medical Imaging, Medical School, University of Pecs, Pecs, Hungary
[e] Department of Biochemistry and Immunology, Lillebaelt Hospital, University Hospital of Southern Denmark, Vejle, Denmark
[f] Department of Regional Health Research, Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark
[g] Department of Neurology, Odense University Hospital, Odense, Denmark
[h] Department of Clinical Research, University of Southern Denmark, Odense, Denmark

ARTICLE INFO

Keywords:
Multiple sclerosis
NAWM
Functional connectivity
fMRI
Resting-state
Diffusion
MR spectroscopy
Osteopontin
Neurofilament

ABSTRACT

Background: Osteopontin (OPN) is a proinflammatory biomarker, and neurofilament light chain (NFL) levels reflect axonal damage. Resting-state functional MRI (rs-fMRI) defines brain networks during wakeful rest.
Objective: To examine, if levels of OPN and NFL are associated on the long term with (i) lesion evolution, (ii) changes in normal-appearing white matter (NAWM) microstructure and (iii) functional connectivity in multiple sclerosis (MS).
Methods: Concentration of NFL and OPN in the blood and CSF were related to MRI findings 10.3 ± 2.8 years later in 53 patients with MS. NFL was examined by Simoa method, OPN by ELISA. Lesion volume in the brain and cervical spinal cord was examined by 3D FLAIR images. Voxel-wise images of fractional anisotropy (FA), axial diffusivity (AD), mean diffusivity (MD), and radial diffusivity (RD) were examined by tract-based spatial statistics corrected for gender, age and lesion volume. Metabolites were examined by single-voxel MR-spectroscopy in the NAWM. Fifty-five default mode network connections were examined by rs-fMRI corrected for gender, age, MS subtype and current therapy as covariates.
Results: While NFL in paired serum and CSF positively correlated ($p = 0.019$), there was no correlation between serum and CSF OPN. Higher OPN levels in the CSF but not in the serum showed association with increased brain WM lesion volume ($p = 0.009$) in 10.3 ± 2.8 years. Higher OPN in the CSF was associated with reduced FA, increased MD, and reduced RD in different NAWM areas 10.3 ± 2.8 years later. Higher OPN in the serum and CSF were associated with increased connectivity strength between the medial prefrontal cortex (MPFC) and other regions except with inferior parietal lobule. NFL in the CSF and in the serum was associated with decreased connectivity strength except for ventral MPFC-hippocampal formation. Neither serum OPN nor NFL at the time of the MRI were associated with functional connectivity changes.
Conclusion: While serum NFL levels reflects CNS production, OPN in serum and CSF may have different cellular sources. OPN within the CSF but not in the serum may forecast development of lesions and microstructural abnormalities in 10 years, indicating the detrimental role of CNS inflammation on the long-term. Although both OPN and NFL in the CSF were associated with functional connectivity changes in 10 years, NFL was associated with decreased strength possibly indicating general axonal loss. In contrast, the positive association of OPN levels in the CSF with increased connectivity strength in 10 years may point to adaptive re-organization due to inflammatory WM lesions and microstructural changes.

* Corresponding author at: Department of Neurology, Odense University Hospital, J.B. Winslows Vej 4, 5000 Odense, Denmark.
E-mail address: zsolt.illes@rsyd.dk (Z. Illes).

https://doi.org/10.1016/j.msard.2021.102923
Received 3 February 2021; Received in revised form 18 March 2021; Accepted 21 March 2021
Available online 24 March 2021

1. Introduction

MRI is a sensitive tool for detecting tissue abnormalities in the central nervous system (CNS) related to multiple sclerosis (MS). The brain-related wide-spread aspects include focal lesions of the white matter (WM) and gray matter (GM), diffuse microstructural abnormalities of the WM, irreversible tissue loss, metabolic changes of the normal-appearing white matter (NAWM), and functional abnormalities measured by functional MRI (fMRI) (Poloni et al., 2011; Rocca et al., 2020).

The functional connectivity within the brain is defined as the coherence in the activity between different cerebral regions under a specific task or in rest. Connectivity analysis based on resting-state functional MRI (rs-fMRI) shows several patterns, defining resting state brain networks (Rosazza and Minati, 2011). One such network is the default mode network (DMN). The main components of the DMN are the posterior cingulate cortex/precuneus, medial prefrontal, and inferior parietal cortices (Broyd et al., 2009). Focal damage to the WM and GM in MS patients is likely to disrupt brain network connections within cortical and subcortical networks (Basile et al., 2014). Default-mode network was reported to be affected by MS pathology both structurally and functionally (Rocca et al., 2010). DMN disruption in MS was associated with a reduced cognitive performance (Rocca et al., 2010), depression (Bonavita et al., 2017), and fatigue (Høgestøl et al., 2019).

Osteopontin (OPN) produced by various immune cells promotes pro-inflammatory cytokine production of Th1 cells, regulates Th17 responses, and inhibits IL-10 production and Th2 polarization, all indicated in the pathogenesis of MS (Rittling and Singh, 2015). Enhanced OPN expression was found in active MS lesions, in the WM surrounding the lesions, and OPN levels in blood and cerebrospinal fluid (CSF) of MS patients are increased (Agah et al., 2018; Chabas et al., 2001).

Neurofilament light chain (NFL) is an emerging biomarker in MS: increased CSF concentrations are reflected by elevated levels in the blood if measured by a sensitive method, Simoa (Kuhle et al., 2016). NFL concentration is elevated at the time of diagnosis and increases during relapse and when new lesions are detected by MRI (Disanto et al., 2017a). Treatment with disease modifying therapies (DMTs) reduces NFL in the CSF and blood (Kuhle et al., 2019; Sejbaek et al., 2019).

In this study, we examined, if OPN and NFL in the serum and CSF predict microstructural, metabolic, and functional abnormalities of the CNS in 10 years measured by MRI; and if the serum level of these biomarkers correlates with such MRI changes in a cross-sectional study.

2. Materials and methods

2.1. Subjects and samples

Fifty-three patients (34 females, age range 20 – 68 years) diagnosed with MS according to the 2017 modified McDonald diagnostic criteria (Thompson et al., 2018) participated in the study (Table 1).

CSF ($n = 33$) and serum ($n = 22$) samples were collected 10.3 ± 2.8 years before MRI, and aliquots were kept at −80 °C. A new serum sample was taken at the day of MRI from each patient ($n = 53$).

The study was conducted according to the World Medical Association Declaration of Helsinki and approved by the Regional Ethical Committee of the University of Pecs (7068-PTE 2018). All patients signed written informed consent.

2.2. Measurement of osteopontin in serum and CSF

We used a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kit (Human Osteopontin DuoSet ELISA, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Serum samples were diluted 1:25 and CSF samples were diluted 1:100 for OPN analysis. All samples were run in duplicates. Optical density was detected at 450 nm using an iEMS MF microphotometer (Thermo Labsystem, Beverly MA, USA). The detection limit for the assay was 62.5 pg/mL.

Table 1
Clinical characteristics of MS patients.

Characteristics	Number of patients, mean±SD or median(IQR)
Demographics	
Number of patients	53
Disease duration (years)	14.5 ± 8.6
Age at onset (years)	31.1 ± 9.6
Sex (male/female)	19/34
Years between CSF examination and MRI	10.3 ± 2.8
Disease type (number of patients)	
PPMS	7 (13%)
SPMS	11 (21%)
RRMS	35 (66%)
EDSS	
At time of CSF examination	2(1.125–2)
At time of MRI	2(1–5.25)
Mental health	
Beck Depression Inventory-II	6(4–13.75)
State-Trait Anxiety Inventory (S)	38.42±11.23
State-Trait Anxiety Inventory (T)	37(32.25–44)
DMT at the time of MRI	
None	17 (32%)
Interferon-beta	12 (23%)
Fingolimod	5 (9%)
Dimethyl fumarate	4 (7.5%)
Teriflunomide	4 (7.5%)
Glatiramer acetate	8 (15%)
Other (alemtuzumab, ocrelizumab, azathioprine)	3 (6%)

Normally distributed data are reported as mean ± SD, while non-normally distributed data are reported as median (25–75% interquartile range). PPMS: primary-progressive multiple sclerosis, SPMS: secondary-progressive multiple sclerosis, RRMS: relapsing-remitting multiple sclerosis, EDSS: Expanded Disability Status Scale, CSF: cerebrospinal fluid, DMT: disease modifying therapy.

2.3. Measurement of NFL in serum and CSF

A commercially available NFL kit (Quanterix©, Lexington, MA, USA) for the Single Molecule Array (Simoa) HD-1 Analyzer (Quanterix) was used to quantify NFL light chain in the serum and CSF according to the manufacturer's procedure. In-house serum and CSF pools were used as internal controls and included in each assay for evaluating assay performance. The total coefficient of variation was <12%. Lower limit of detection was 0.038 pg/mL and lower limit of quantification was 0.174 pg/mL.

2.4. Magnetic resonance imaging

All subjects were scanned using the same 3T MRI scanner (MAGNETOM Prisma^{Fit}, Siemens Healthineers, Erlangen, Germany). The prospective MRI study protocol included the following sequences: 3D T1 magnetization-prepared rapid acquisition with gradient echo (MPRAGE), 3D fluid-attenuated inversion recovery (FLAIR), diffusion tensor imaging (DTI), single voxel Point RESolved Spectroscopy (MRS), phase-sensitive inversion recovery (PSIR) imaging of the cervical spine region and resting-state functional MRI (rs-fMRI) with field mapping to reduce image distortions due to B0 inhomogeneities. Sequence parameters are detailed in the Supplementary Materials.

2.5. Tract-based spatial statistics (TBSS) analysis

Voxel-wise images of fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD) and radial diffusivity (RD) were calculated using FMRIB's diffusion toolbox by fitting a diffusion tensor model to the pre-processed diffusion data at each brain voxel (Smith et al., 2004).

Voxel-wise statistical analyses of diffusion data were carried out using TBSS v1.2 (Smith et al., 2006). The analysis steps of DTI pre-processing and TBSS are detailed in the Supplementary Materials.

2.6. Segmentation of cerebral white matter and cervical spine lesions

Cerebral white matter lesions were segmented on 3D FLAIR images using the lesion prediction algorithm (Schmidt, 2017) as implemented in the LST toolbox version 3.0.0 (Lesion Segmentation Toolbox, www.statistical-modelling.de/lst.html) for SPM (SPM12). Cervical spine lesions were manually segmented on sagittal PSIR images using 3DSlicer (4.10.2 r28257). Level tracing option was used within the editor and the resulting labels were manually corrected. Label statistics were used to export the number of lesions and the volume of each lesion for statistical evaluation.

2.7. rs-fMRI evaluation

rs-fMRI data were pre-processed using DPARSF 5.0, part of DPABI (V4.3_200401, http://rfmri.org/dpabi) (Yan et al., 2016) and SPM 12. Functional connectivity was automatically calculated between the 11 regions of interest (ROIs) of the Default Mode network (DMN) based on the Andrews-Hannah DMN atlas (Andrews-Hanna et al., 2010) along with the amplitude of low-frequency fluctuation (ALFF) maps for all subjects. The z-standardized functional connectivity values between the predefined ROIs were exported for further statistical analyses. Fifty-five connections were examined altogether ($\frac{n*(n-1)}{2}$). See Table 2 for the names, abbreviations, and MNI coordinates of DMN ROIs and Supplementary Materials for further details on evaluation.

2.8. Single-voxel MR spectroscopy

Water-scaled spectroscopy data were evaluated using LCModel Version 6.2 (Provencher, 1993). The absolute metabolite concentrations of total N-Acetylaspartate (tNAA), total Choline (tCho), total Creatine (tCr), and myo-inositol (Ins) were calculated. Further details are discussed in Supplementary Materials.

2.9. Statistical analysis

For TBSS and ALFF analyses, voxel-wise statistics were performed using a permutation-based non-parametric analysis.

All other statistical analyses were performed using SPSS (IBM Corp., Version 25.0. Armonk, NY). The statistical analysis is detailed in the Supplementary materials.

Table 2
Default mode network ROIs.

Region	Abbreviation	MNI x	MNI y	MNI z
Anterior medial prefrontal cortex	aMPFC	−6	52	−2
Posterior cingulate cortex	PCC	−8	−56	26
Dorsomedial prefrontal cortex	dMPFC	0	52	26
Temporal parietal junction	TPJ	−54	−54	28
Lateral temporal cortex	LTC	−60	−24	−18
Temporal pole	TempP	−50	14	−40
Ventromedial prefrontal cortex	vMPFC	0	26	−18
Posterior inferior parietal lobule	pIPL	−44	−74	32
Retrosplenial cortex	Rsp	−14	−52	8
Parahippocampal cortex	PHC	−28	−40	−12
Hippocampal formation	HF	−22	−20	−26

Names, abbreviations, and Montreal Neurological Institute (MNI) coordinates (mm) of the default mode network ROIs. Spherical ROIs were generated with a radius of 4 mm centered at the coordinates listed above. ROIs are based on Andrews-Hannah DMN atlas [26].

3. Results

3.1. Patient demographics

During the time between CSF collection and MRI, EDSS has significantly increased ($p = 0.046$, Wilcoxon Signed Rank Test) in the patient population, and 44% of patients showed disability progression. The mean number of relapses during the same period, was 3.72 ± 2.49. At the time of MRI, 66% of the patients had relapsing-remitting MS (RRMS), 21% secondary progressive MS (SPMS), and 13% primary progressive MS, but no patients had clinical and/or MRI activity.

3.2. No correlation between CSF and serum OPN concentrations in contrast to NFL

We found correlation between the median NFL level in the CSF (1406 pg/mL, 447–3163) and the paired serum (17.85 pg/mL, 9.4–28.8) (Spearman's ρ=0.576, $p = 0.019$). Median NFL level in serum at the time of MRI was 9.3 (7.8–16.1) pg/mL.

The median OPN level in the CSF (246.36 ng/mL, 164.5–439.5), and paired serum (12.52 ng/mL, 6.8–16.7) showed no correlation. Median OPN serum level at the time of MRI was 9.8 (6.2–18.8) ng/mL.

3.3. Association of OPN with lesion volumes in brain WM but not in the cervical spinal cord

The LST segmented median total lesion volume in the cerebral white matter was 2774 mm^3 (701.75–7713.25). NFL concentrations in CSF and paired serum before MRI, and in serum at the time of MRI did not show significant association with the LST segmented total lesion volume. Higher baseline CSF OPN levels were associated with increased total brain WM lesion volume over a mean of 10 years ($p = 0.009$, $t = 2.877$).

The median segmented lesion volume in the cervical spine was 300 mm^3 (88–652), and 57% of patients had multiple lesions. Neither OPN, nor NFL showed any association with the number of lesions, the total lesion volume, or the number of lesions larger than 300 mm^3.

3.4. Higher OPN but not NFL in the CSF predicted microstructural changes in the NAWM

Gender, age, and total cerebral lesion volume were included as nuisance variables. CSF OPN levels were significantly associated with microstructural alterations in the NAWM 10.3 ± 2.8 years later: higher levels were associated with reduced FA, increased MD, and reduced RD affecting left superior and inferior longitudinal fasciculi, external capsule, forceps minor (genu of corpus callosum), anterior corona radiata, and to a smaller extent the right inferior longitudinal fasciculus and corona radiata (**Fig. 1**). OPN levels in the sera showed no association with the microstructure of the WM skeleton.

Baseline serum/CSF NfL levels were not correlated with alterations in the NAWM found at 10.3 ± 2.8 years later, neither with the serum at the time of the MRI.

3.5. Trend of association between CSF OPN and single-voxel spectroscopy

OPN measured from CSF obtained 10.3 ± 2.8 years earlier was associated with tCr ($t = 2.578$, $p = 0.018$) and Ins ($t = 2.796$, $p = 0.0129$) concentrations, but none survived multiple comparisons correction. tCho was strongly associated with DMT applied (**Fig. 2**).

OPN and NFL levels in the sera obtained 10.3 ± 2.8 years earlier or at the time of MRI, as well as CSF NFL showed no association with metabolic changes in the NAWM voxel.

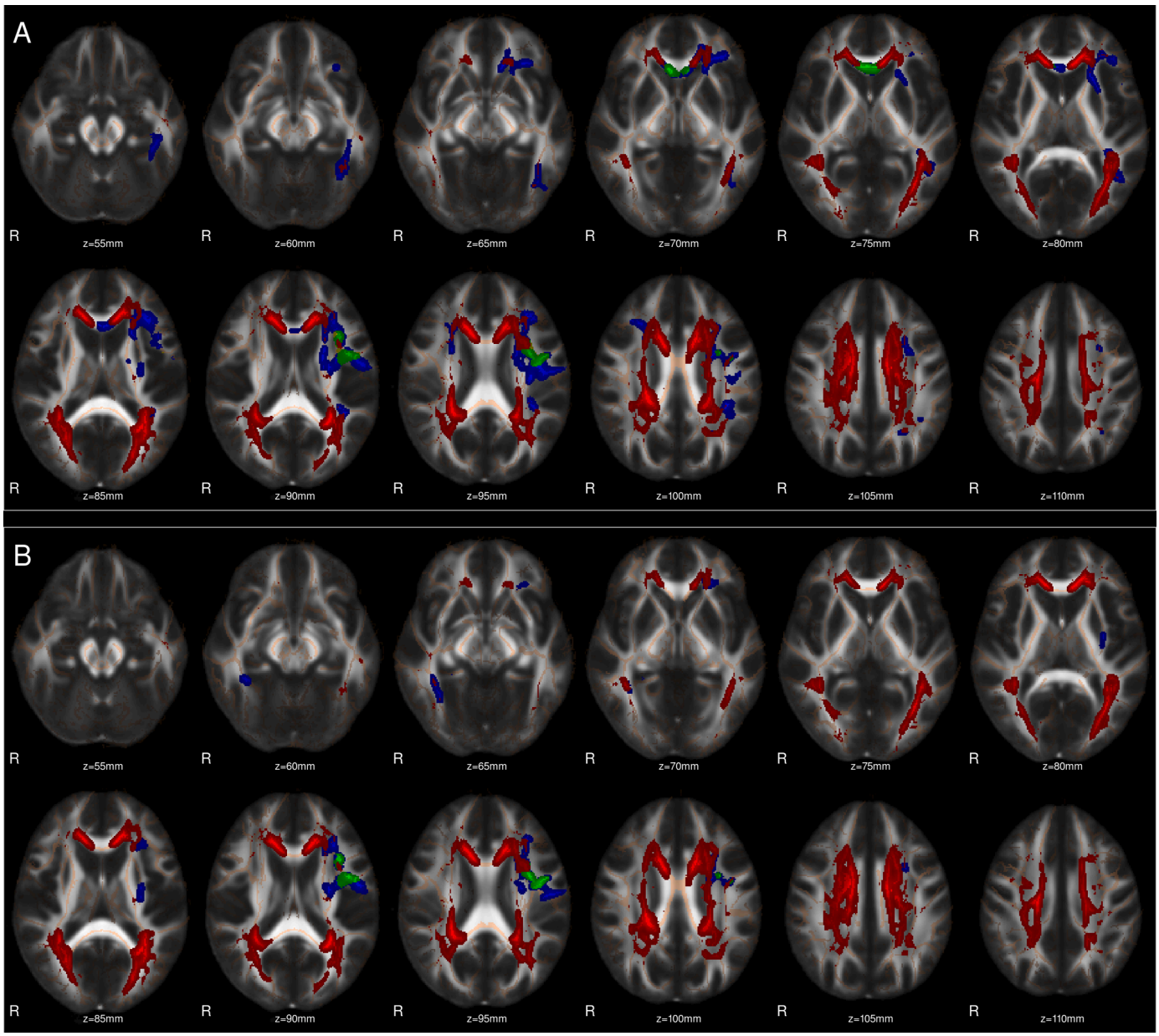


Fig. 1. Tract-based spatial statistics analysis of the white matter in patients with multiple sclerosis.
Upper table (A): Areas with significant associations (corrected $p < 0.05$) between CSF NFL levels and fractional anisotropy (FA) and mean diffusivity (MD) values measured ~10 years later are shown in yellow and blue, respectively. Overlapping areas (FA+MD) are depicted in green. A lesion mask (red) is overlaid on top of all other layers to mask associations within multiple sclerosis (MS) lesions. Lower table (B): Areas with significant associations (corrected $p < 0.05$) between CSF NFL levels and radial diffusivity (RD) values measured ~10 years later are shown in blue. Overlapping areas (FA+MD+RD) are depicted in green. A lesion mask (red) is overlaid on top of all other layers to mask associations within MS lesions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. OPN and NFL are associated with functional connectivity state after 10 years

Permutation-based non-parametric tests showed no significant association between ALFF and soluble biomarkers, irrespective of sample type and time of collection.

However functional connectivity between the 11 default mode network nodes showed several associations with OPN and NFL after correcting for age, gender, anxiety, depression, disease type and current DMT.

Baseline CSF NfL concentration was associated with functional connectivity strength at year 10.3 ± 2.8 of follow-up regarding the following connections: PHC-pIPL (t=−3.611, p = 0.002), aMPFC-pIPL (t=−2.483, p = 0.025), vMPFC-HF (t = 2.413, p = 0.028), and TempP-TPJ (t=−2.138, p = 0.048) (Fig. 3A, Table 3). NFL in the paired serum was associated with functional connectivity between vMPFC-pIPL (t=−3.831, p = 0.004). Serum NFL collected at the time of MRI showed no significant associations.

OPN measured from CSF 10.3 ± 2.8 years before MRI showed association with TempP-vMPFC functional connectivity (t = 2.435, p = 0.026), while serum OPN levels measured in paired serum showed numerous associations with functional connectivity strengths between the nodes of DMN (Fig. 3B, Table 3). Serum OPN levels at the time of the MRI showed no significant associations.

Functional connectivity between vMPFC-pIPL and vMPFC-HF were related to both OPN and NFL concentrations measured 10.3 ± 2.8 years

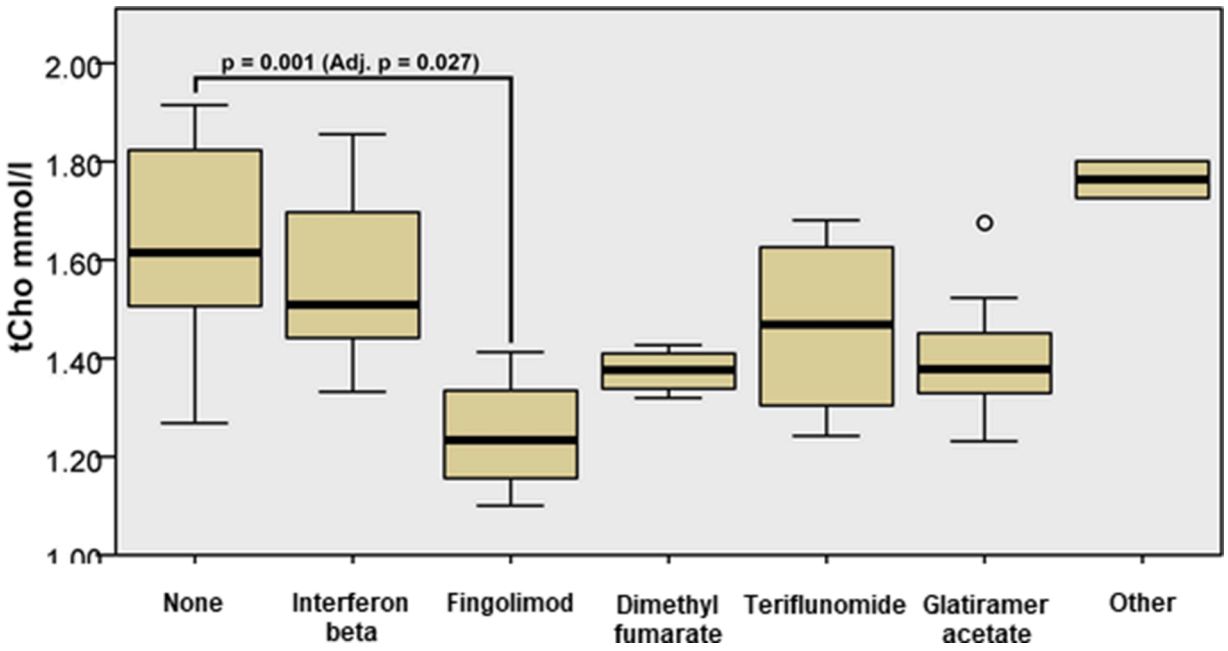


Fig. 2. Effect of therapy on total Choline levels measured in multiple sclerosis patients.
Single-voxel MR spectroscopy in multiple sclerosis (MS) patients measured from centrum semiovale normal-appearing white matter. Horizontal line: median; box: interquartile range (25–75%), whiskers are set to minimum and maximum. Outliers are marked with°. Kruskal-Wallis Test ($p = 0.004$). Post-hoc pairwise comparisons revealed a significant difference between patients without disease modifying therapy and patients treated with fingolimod, $p = 0.001$ (Adjusted $p = 0.027$, after Bonferroni correction). tCho: total choline.

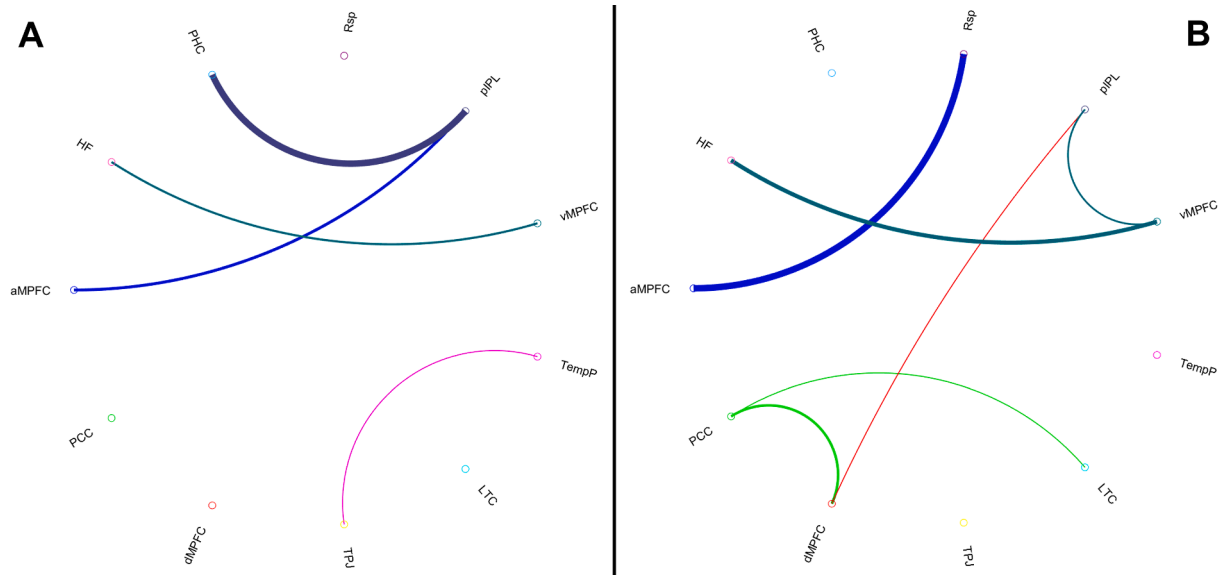


Fig. 3. Association between NFL and OPN concentration and functional connectivity strengths within the default mode network.
A, Associations between NFL measured from CSF sample collected ~10 years before MRI and functional connectivity between the nodes of DMN are depicted. B, Associations between OPN measured from serum sample collected ~10 years before MRI and functional connectivity between the nodes of DMN are depicted. Only the significant associations are shown, line thickness corresponds to the 1-"*p*-value". Statistical data are summarized in Table 3.

before MRI (Table 3).

3.7. MRI and biomarker outcomes are associated with EDSS and EDSS changes

Baseline EDSS was significantly associated to lesion load (volume) assessed on FLAIR images ($p = 0.05$) and cervical spine lesion volume assessed on PSIR images ($p = 0.016$), controlling for age, gender and total intracranial volume. EDSS change showed no association with either volumetric measures or lesion load.

EDSS baseline also showed significant association with the following connection strengths aMPFC-TempP ($p = 0.025$), PCC-TempP ($p = 0.046$), and vMPFC-PHC ($p = 0.003$), adjusted for age, gender, current DMTs, anxiety, and depression. EDSS change was not associated with functional connectivities.

Neither EDSS baseline, nor EDSS change showed any significant associations with white matter microstructure after correcting for age and gender. However, EDSS baseline showed nearly significant associations

Table 3
Significant associations between functional connectivity within DMN and soluble biomarkers.

Soluble biomarker concentration	DMN nodes	*t*	*p*
NfL CSF	PHC-pIPL	−3.611	0.002
	aMPFC-pIPL	−2.483	0.025
	vMPFC-HF	2.413	0.028
	TempP-TPJ	−2.138	0.048
NfL Serum at LP	vMPFC-pIPL	−3.831	0.004
NfL Serum at MRI	–		
OPN CSF	TempP-vMPFC	2.435	0.026
OPN serum at LP	vMPFC-pIPL	−2.658	0.038
	vMPFC-HF	3.506	0.013
	aMPFC-Rsp	3.831	0.009
	dMPFC-PCC	3.000	0.024
	dMPFC-pIPL	2.556	0.043
	PCC-LTC	2.525	0.045
OPN serum at MRI	–		

Z-standardized correlation strength between DMN nodes were included in multiple linear regression as dependent variable. Model was corrected for age, gender, anxiety, depression, disease type and current therapy. Current therapy was a significant predictor in all models. LP = lumbar punction, all other abbreviations are reported in Table 2.

with mean diffusivity (MD) in the left forceps major (p-values between 0.052–0.06).

Neither EDSS at the time of MRI nor EDSS change showed significant correlation with baseline NfL in the serum and in the CSF, and with baseline OPN in the serum and in the CSF, respectively.

4. Discussion

OPN is regarded as a proinflammatory biomarker produced by both peripheral immune cells and CNS resident cells (Rittling and Singh, 2015;'Niino and Kikuchi, 2011; Hammond et al., 2019; Neumann et al., 2014a). NFL is primarily a marker of axonal destruction in both neuroinflammatory and neurodegenerative conditions, and its CSF level reflects acute neuronal and axonal damage in MS (Khalil et al., 2018). Our study controlled for age, sex, MS subtype, depression, anxiety and treatments examined the so far unaddressed questions of OPN and NFL association with (i) microstructural and (ii) metabolic changes in the NAWM, and (iv) functional connectivity in 10 years.

Our results indicate that OPN and NFL may be related to different aspects of developing MS pathology. OPN measured from CSF was associated with extensive changes in NAWM microstructure, and changes in functional connectivity at year 10.3 ± 2.8 of follow-up. NFL on the other hand showed no such association with NAWM microstructural changes, albeit showed a strong association with the functional connectivity of two main DMN nodes, the MPFC and the pIPL. Thus, while CNS inflammation in MS contributes to lesion evolution and extensive abnormalities in the NAWM in a decade associated with changes in functional connectivity, the inflammation-related axonal damage affects global functional changes on the long term.

The association of OPN levels in the CSF with increased lesion volume in 10 years is not surprising, as OPN produced by both infiltrating peripheral lymphocytes and CNS resident cells may contribute to lesion evolution. Elevation of CSF and peripheral blood OPN levels have been detected in RRMS and SPMS, and higher OPN levels in CSF were measured in patients with active MS compared to patients with stable disease (Agah et al., 2018). Interferon beta, a platform therapy in MS regulates OPN expression (Chen et al., 2009). We also found that high levels of OPN in the CSF were associated with reduced FA, increased MD and reduced RD at different CNS sites in 10 years indicating that inflammation also affects microstructural changes in the NAWM on the long term. Correlation of OPN levels with NAWM changes was also supported by the observed trend of association between OPN levels in the CSF and metabolic changes (tCr and Ins) in an NAWM voxel. Currently applied DMT was a highly significant predictor in the statistical models used to track metabolic changes. In a clinical trial of patients with progressive MS, natalizumab that prevents the migration of inflammatory lymphocytes reduced the level of OPN (Christensen et al., 2014). Besides peripheral lymphocytes, OPN expressed by microglia and reactive astrocytes in the NAWM distant from MS lesions (Chabas et al., 2001; Neumann et al., 2014b) may contribute to such microstructural abnormalities. In the insulted brain, OPN recruits microglia, macrophages and astrocytes, modulating inflammatory responses and attenuating secondary neurodegeneration (Rabenstein et al., 2016; Riew et al., 2019). Expression of the OPN gene *SPP1* is especially high in activated microglia (Hammond et al., 2019). OPN promotes progression and relapse in MS by enhancing the survival of activated T cells (Hur et al., 2007).

NFL concentration in the CSF was associated with reduced functional connectivity between PHC-pIPL and aMPFC-pIPL, while increased functional connectivity between the vMPFC and hippocampus. Serum OPN levels 10.3 ± 2.8 years before MRI also correlated positively with the vMPFC-hippocampus functional connectivity strength. Two more general observations can be made based on Table 3. First, neither NFL, nor OPN measured from serum at the day of MRI showed any significant association with the examined functional connectivity strengths. This may indicate that such changes need time to develop, and although effect of inflammation on network functioning is not immediate, it has long-term consequences. And second, while NFL concentration showed mainly negative associations with functional connectivity strength between DMN nodes (except for vMPFC-HF), OPN concentrations showed mainly positive associations (except for vMPFC-pIPL). The negative associations related to NFL may reflect the general loss of axons and connection in the brain, while the positive associations of OPN may be related to reorganization due to the inflammatory damage.

Associations between the functional connectivity of DMN and the biomarker concentrations measured from samples collected approximately a decade ago point towards the anterior DMN, especially the MPFC. The functional connectivity of the MPFC showed characteristic associations with OPN or NFL concentrations. Except for the MPFC-pIPL connections, all other MPFC related connections showed a positive association with the measured biomarker concentrations. This indicates that 10 years after the sample collection, patients with higher OPN or NFL concentrations showed stronger functional connectivities between MPFC and other regions (hippocampus, posterior cingulum, inferior parietal lobule, and retrosplenial cortex). This finding can be partially explained by plasticity, which allows an adaptive and effective reorganization to limit impairment. The recruitment of MPFC is a form of adaptive brain plasticity in MS to compensate for relative deficits in information processing. Indeed, MS patients had significantly larger activation in the MPFC compared to healthy controls in Stroop fMRI test, although performance was not different (Parry et al., 2003). The neurobiological background behind this adaptive change is probably driven by the 'unmasking' of latent pathways (Parry et al., 2003). We may speculate that higher OPN and NFL concentrations predict worse outcome and enhanced progression on the long run causing higher information processing deficiency, resulting in enhanced activation and more effective functional connectivity of the MPFC to alleviate the deficit. OPN may be associated with WM damage (Selvaraju et al., 2004), which is supported by our data: CSF OPN levels were related to a wide-spread alterations in the NAWM of the left superior and inferior longitudinal fasciculi, external capsule, forceps minor (genu of corpus callosum) and anterior corona radiata, indicating myelin loss.

Although we found correlation between NFL levels in blood and CSF similarly to recent studies (Disanto et al., 2017b; Sejbaek et al., 2019),

OPN levels in the two compartments did not correlate. While NFL in the serum reflects production in the CNS, concentration of OPN in the serum indicates both peripheral and possibly CNS sources. Since we did not find association between serum OPN and microstructural abnormalities, our data may suggest that OPN produced within the CNS/CSF by infiltrating lymphocytes and resident cells may be important in the development of such alterations.

In conclusion, our data indicate that both OPN and NFL levels in the CSF predict changes in functional connectivity in 10 years, while OPN also predict microstructural abnormalities in the NAWM. These data add additional layers to the predictive role of NFL in MS outcome measures and strengthen the early introduction of DMTs in order to prevent the long-term effect of inflammation.

Author contributions

Gergely Orsi: Conceptualization, Methodology, Data curation, Formal analysis, Writing- Original draft preparation, Funding acquisition, Writing-Reviewing and Editing.

Tamas Cseh; Conceptualization, Methodology, Data curation, Writing- Reviewing and Editing.

Zsofia Hayden: Data curation.

Gabor Perlaki: Data curation.

Szilvia Anett Nagy: Data curation.

Omar Giyab: Data curation.

Dorte Aalund Olsen: Data curation, Methodology.

Jonna Skov Madsen: Data curation, Methodology.

Timea Berki: Conceptualization, Methodology, Data curation, Writing- Reviewing and Editing.

Zsolt Illes: Conceptualization, Methodology, Data curation, Writing-Reviewing and Editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The Author(s) declare(s) that there is no conflict of interest.

Acknowledgements

This study was supported by grants from a joint grant by Odense University Hospital and Rigshospital (to ZI), EFOP-3.6.2-16-2017-00008 „The role of neuro-inflammation in neurodegeneration: from molecules to clinics" and the Institutional Excellence Program for the Higher Education II within the framework of the 5th thematic program and the 2020-4.1.1-TKP2020 - FIKP III (to GO). GO was supported by the University of Pécs Medical School Research Fund (KA-2017-06).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2021.102923.

References

Agah, E., Zardoui, A., Saghazadeh, A., Ahmadi, M., Tafakhori, A., Rezaei, N., 2018. Osteopontin (OPN) as a CSF and blood biomarker for multiple sclerosis: a systematic review and meta-analysis. PLoS ONE 13, e0190252. https://doi.org/10.1371/journal.pone.0190252.

Andrews-Hanna, J.R., Reidler, J.S., Sepulcre, J., Poulin, R., Buckner, R.L., 2010. Functional-anatomic fractionation of the brain's default network. Neuron 65, 550–562. https://doi.org/10.1016/j.neuron.2010.02.005.

Basile, B., Castelli, M., Monteleone, F., Nocentini, U., Caltagirone, C., Centonze, D., Cercignani, M., Bozzali, M., 2014. Functional connectivity changes within specific networks parallel the clinical evolution of multiple sclerosis. Mult. Scler. J. 20, 1050–1057. https://doi.org/10.1177/1352458513515082.

Bonavita, S., Sacco, R., Esposito, S., d'Ambrosio, A., Della Corte, M., Corbo, D., Docimo, R., Gallo, A., Lavorgna, L., Cirillo, M., Bisecco, A., Esposito, F., Tedeschi, G., 2017. Default mode network changes in multiple sclerosis: a link between depression and cognitive impairment? Eur. J. Neurol. 24, 27–36. https://doi.org/10.1111/ene.13112.

Broyd, S.J., Demanuele, C., Debener, S., Helps, S.K., James, C.J., Sonuga-Barke, E.J.S., 2009. Default-mode brain dysfunction in mental disorders: a systematic review. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2008.09.002.

Chabas, D., Baranzini, S.E., Mitchell, D., Bernard, C.C.A., Rittling, S.R., Denhardt, D.T., Sobel, R.A., Lock, C., Karpuj, M., Pedotti, R., Heller, R., Oksenberg, J.R., Steinman, L., 2001. The influence of the proinflammatory cytokine, osteopontin, on autoimmue demyelinating desease. Science (80-.) 294, 1731–1735. https://doi.org/10.1126/science.1062960.

Chen, M., Chen, G., Nie, H., Zhang, X., Niu, X., Zang, Y.C.Q., Skinner, S.M., Zhang, J.Z., Killian, J.M., Hong, J., 2009. Regulatory effects of IFN-β on production of osteopontin and IL-17 by CD4+ T Cells in MS. Eur. J. Immunol. 39, 2525–2536. https://doi.org/10.1002/eji.200838879.

Christensen, J.R., Ratzer, R., Börnsen, L., Lyksborg, M., Garde, E., Dyrby, T.B., Siebner, H.R., Sorensen, P.S., Sellebjerg, F., 2014. Natalizumab in progressive MS: results of an open-label, phase 2A, proof-of-concept trial. Neurology 82, 1499–1507. https://doi.org/10.1212/WNL.0000000000000361.

Disanto, G., Barro, C., Benkert, P., Naegelin, Y., Schädelin, S., Giardiello, A., Zecca, C., Blennow, K., Zetterberg, H., Leppert, D., Kappos, L., Gobbi, C., Kuhle, J., 2017a. Serum Neurofilament light: a biomarker of neuronal damage in multiple sclerosis. Ann. Neurol. 81, 857–870. https://doi.org/10.1002/ana.24954.

Disanto, G., Barro, C., Benkert, P., Naegelin, Y., Schädelin, S., Giardiello, A., Zecca, C., Blennow, K., Zetterberg, H., Leppert, D., Kappos, L., Gobbi, C., Kuhle, J., 2017b. Serum Neurofilament light: a biomarker of neuronal damage in multiple sclerosis. Ann. Neurol. 81, 857–870. https://doi.org/10.1002/ana.24954.

Hammond, T.R., Dufort, C., Dissing-Olesen, L., Giera, S., Young, A., Wysoker, A., Walker, A.J., Gergits, F., Segel, M., Nemesh, J., Marsh, S.E., Saunders, A., Macosko, E., Ginhoux, F., Chen, J., Franklin, R.J.M., Piao, X., McCarroll, S.A., Stevens, B., 2019. Single-cell RNA sequencing of microglia throughout the mouse lifespan and in the injured brain reveals complex cell-state changes. Immunity 50. https://doi.org/10.1016/j.immuni.2018.11.004, 253-271.e6.

Høgestøl, E.A., Nygaard, G.O., Alnæs, D., Beyer, M.K., Westlye, L.T., Harbo, H.F., 2019. Symptoms of fatigue and depression is reflected in altered default mode network connectivity in multiple sclerosis. PLoS ONE 14, 1–14. https://doi.org/10.1371/journal.pone.0210375.

Hur, E.M., Youssef, S., Haws, M.E., Zhang, S.Y., Sobel, R.A., Steinman, L., 2007. Osteopontin-induced relapse and progression of autoimmune brain disease through enhanced survival of activated T cells. Nat. Immunol. 8, 74–83. https://doi.org/10.1038/ni1415.

Khalil, M., Teunissen, C.E., Otto, M., Piehl, F., Sormani, M.P., Gattringer, T., Barro, C., Kappos, L., Comabella, M., Fazekas, F., Petzold, A., Blennow, K., Zetterberg, H., Kuhle, J., 2018. Neurofilaments as biomarkers in neurological disorders. Nat. Rev. Neurol. 14, 577–589. https://doi.org/10.1038/s41582-018-0058-z.

Kuhle, J., Barro, C., Andreasson, U., Derfuss, T., Lindberg, R., Sandelius, Å., Liman, V., Norgren, N., Blennow, K., Zetterberg, H., 2016. Comparison of three analytical platforms for quantification of the neurofilament light chain in blood samples: ELISA, electrochemiluminescence immunoassay and Simoa. Clin. Chem. Lab. Med. 54 https://doi.org/10.1515/cclm-2015-1195.

Kuhle, J., Kropshofer, H., Haering, D.A., Kundu, U., Meinert, R., Barro, C., Dahlke, F., Tomic, D., Leppert, D., Kappos, L., 2019. Blood neurofilament light chain as a biomarker of MS disease activity and treatment response. Neurology 92. https://doi.org/10.1212/WNL.0000000000007032. E1007–E1015.

Neumann, C., Garreis, F., Paulsen, F., Hammer, C.M., Birke, M.T., Scholz, M., 2014a. Osteopontin is induced by TGF-β2 and regulates metabolic cell activity in cultured human optic nerve head astrocytes. PLoS ONE 9, e92762. https://doi.org/10.1371/journal.pone.0092762.

Neumann, C., Garreis, F., Paulsen, F., Hammer, C.M., Birke, M.T., Scholz, M., 2014b. Osteopontin is induced by TGF-β2 and regulates metabolic cell activity in cultured human optic nerve head astrocytes. PLoS ONE 9, e92762. https://doi.org/10.1371/journal.pone.0092762.

Niino, M., Kikuchi, S., 2011. Osteopontin and multiple sclerosis: an update. Clin. Exp. Neuroimmunol. 2, 33–40. https://doi.org/10.1111/j.1759-1961.2011.00019.x.

Parry, A.M.M., Scott, R.B., Palace, J., Smith, S., Matthews, P.M., 2003. Potentially adaptive functional changes in cognitive processing for patients with multiple sclerosis and their acute modulation by rivastigmine. Brain 126, 2750–2760. https://doi.org/10.1093/brain/awg284.

Poloni, G., Minagar, A., Haacke, E.M., Zivadinov, R., 2011. Recent developments in imaging of multiple sclerosis. Neurologist. https://doi.org/10.1097/NRL.0b013e31821a2643.

Provencher, S.W., 1993. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. Magn. Reson. Med. 30, 672–679. https://doi.org/10.1002/mrm.1910300604.

Rabenstein, M., Vay, S.U., Flitsch, L.J., Fink, G.R., Schroeter, M., Rueger, M.A., 2016. Osteopontin directly modulates cytokine expression of primary microglia and increases their survival. J. Neuroimmunol. 299, 130–138. https://doi.org/10.1016/j.jneuroim.2016.09.009.

Riew, T.-.R., Kim, S., Jin, X., Kim, H.L., Lee, J.-.H., Lee, M.-.Y., 2019. Osteopontin and its spatiotemporal relationship with glial cells in the striatum of rats treated with mitochondrial toxin 3-nitropropionic acid: possible involvement in phagocytosis. J. Neuroinflammation 16, 99. https://doi.org/10.1186/s12974-019-1489-1.

Rittling, S.R., Singh, R., 2015. Osteopontin in immune-mediated diseases. J. Dent. Res. 94, 1638–1645. https://doi.org/10.1177/0022034515605270.

Rocca, M.A., De Meo, E., Filippi, M., 2020. Resting-state fMRI in multiple sclerosis. FMRI 335–353. https://doi.org/10.1007/978-3-030-41874-8_23.

Rocca, M.A., Valsasina, P., Absinta, M., Riccitelli, G., Rodegher, M.E., Misci, P., Rossi, P., Falini, A., Comi, G., Filippi, M., 2010. Default-mode network dysfunction and

cognitive impairment in progressive MS. Neurology 74, 1252–1259. https://doi.org/10.1212/WNL.0b013e3181d9ed91.

Rosazza, C., Minati, L., 2011. Resting-state brain networks: literature review and clinical applications. Neurol. Sci. 32, 773–785. https://doi.org/10.1007/s10072-011-0636-y.

Schmidt, P., 2017. Bayesian inference for structured additive regression models for large-scale problems with applications to medical imaging. LMU München. https://doi.org/10.5282/edoc.20373.

Sejbaek, T., Nielsen, H.H., Penner, N., Plavina, T., Mendoza, J.P., Martin, N.A., Elkjaer, M.L., Ravnborg, M.H., Illes, Z., 2019. Dimethyl fumarate decreases neurofilament light chain in CSF and blood of treatment naïve relapsing MS patients. J. Neurol. Neurosurg. Psychiatry. https://doi.org/10.1136/jnnp-2019-321321 jnnp-2019-321321.

Selvaraju, R., Bernasconi, L., Losberger, C., Graber, P., Kadi, L., Avellana-Adalid, V., Picard-Riera, N., Van Evercooren, A.B., Cirillo, R., Kosco-Vilbois, M., Feger, G., Papoian, R., Boschert, U., 2004. Osteopontin is upregulated during in vivo demyelination and remyelination and enhances myelin formation in vitro. Mol. Cell. Neurosci. 25, 707–721. https://doi.org/10.1016/j.mcn.2003.12.014.

Smith, S.M., Jenkinson, M., Johansen-Berg, H., Rueckert, D., Nichols, T.E., Mackay, C.E., Watkins, K.E., Ciccarelli, O., Cader, M.Z., Matthews, P.M., Behrens, T.E.J., 2006. Tract-based spatial statistics: voxelwise analysis of multi-subject diffusion data. Neuroimage 31, 1487–1505. https://doi.org/10.1016/j.neuroimage.2006.02.024.

Smith, S.M., Jenkinson, M., Woolrich, M.W., Beckmann, C.F., Behrens, T.E.J., Johansen-Berg, H., Bannister, P.R., De Luca, M., Drobnjak, I., Flitney, D.E., Niazy, R.K., Saunders, J., Vickers, J., Zhang, Y., De Stefano, N., Brady, J.M., Matthews, P.M., 2004. Advances in functional and structural MR image analysis and implementation as FSL. NeuroImage. Neuroimage. https://doi.org/10.1016/j.neuroimage.2004.07.051.

Thompson, A.J., Banwell, B.L., Barkhof, F., Carroll, W.M., Coetzee, T., Comi, G., Correale, J., Fazekas, F., Filippi, M., Freedman, M.S., Fujihara, K., Galetta, S.L., Hartung, H.P., Kappos, L., Lublin, F.D., Marrie, R.A., Miller, A.E., Miller, D.H., Montalban, X., Mowry, E.M., Sorensen, P.S., Tintoré, M., Traboulsee, A.L., Trojano, M., Uitdehaag, B.M.J., Vukusic, S., Waubant, E., Weinshenker, B.G., Reingold, S.C., Cohen, J.A., 2018. Diagnosis of multiple sclerosis: 2017 revisions of the McDonald criteria. Lancet Neurol. https://doi.org/10.1016/S1474-4422(17)30470-2.

Yan, C.G., Wang, X.Di, Zuo, X.N., Zang, Y.F., 2016. DPABI: data processing & analysis for (resting-state) brain imaging. Neuroinformatics 14, 339–351. https://doi.org/10.1007/s12021-016-9299-4.