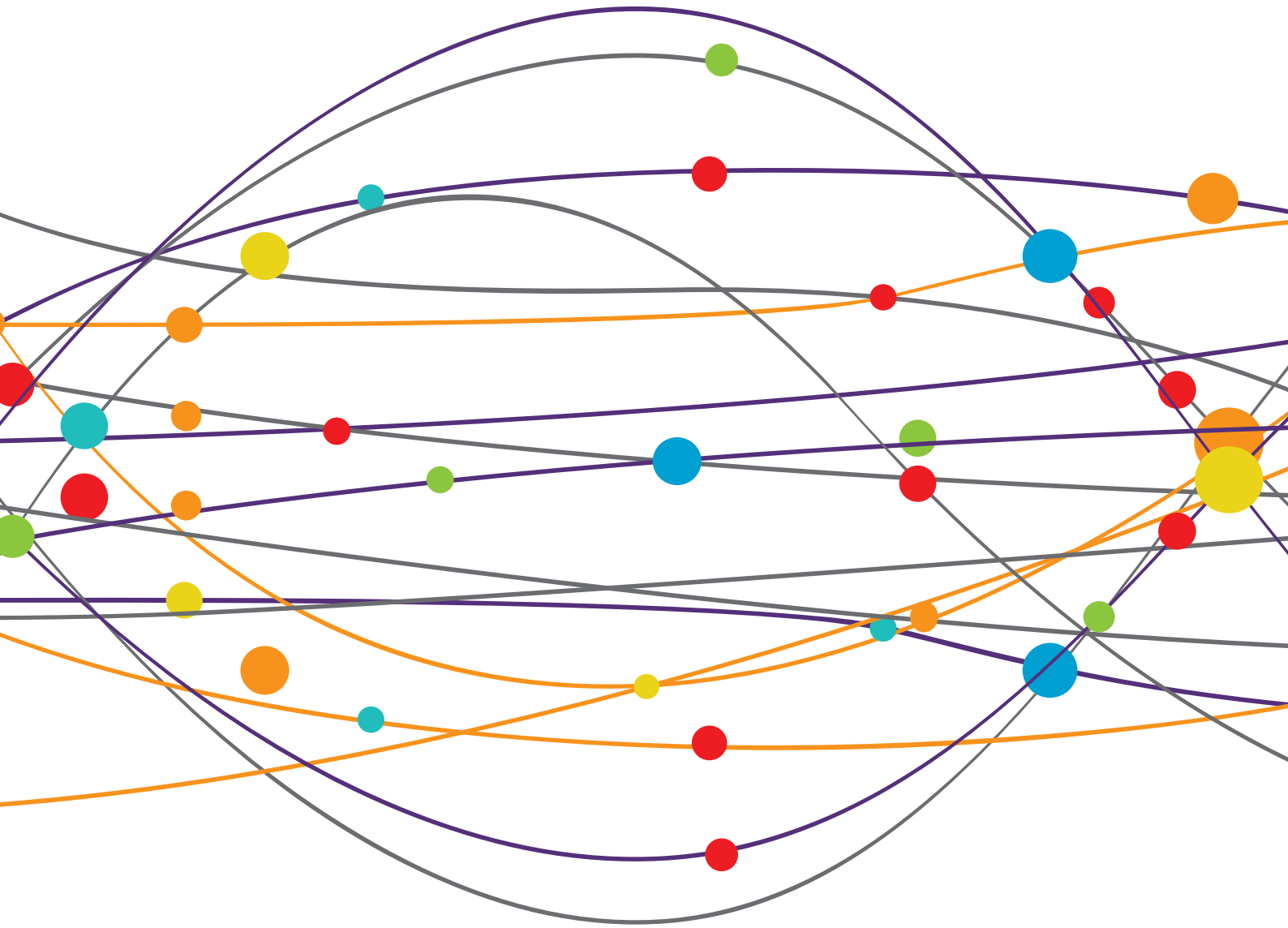
AUTOIMMUNITY AND THE BRAIN: PARANEOPLASTIC NEUROLOGICAL INJURY AND BEYOND

EDITED BY: John Greenlee, Stacey L. Clardy and Christian Vedeler
PUBLISHED IN: Frontiers in Neurology and Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88976-268-2
DOI 10.3389/978-2-88976-268-2

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AUTOIMMUNITY AND THE BRAIN: PARANEOPLASTIC NEUROLOGICAL INJURY AND BEYOND

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Citation: Greenlee, J., Clardy, S. L., Vedeler, C., eds. (2022). Autoimmunity and the Brain: Paraneoplastic Neurological Injury and Beyond. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-268-2

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frontiers | Frontiers in Neurology

EDITORIAL
published: 12 May 2022
doi: 10.3389/fneur.2022.900130

Editorial: Autoimmunity and the Brain: Paraneoplastic Neurological Injury and Beyond

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Keywords: autoimmune neurology, autoimmune encephalitis, paraneoplastic neurological syndromes, tissue culture, animal models, immune checkpoint inhibitors, treatment

OPEN ACCESS

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

Received: *20 March 2022*
Accepted: *28 March 2022*
Published: *12 May 2022*

Citation:
Greenlee JE, Carlson NG, Abbatemarco JR, Herdlevær I, Clardy SL and Vedeler CA (2022) Editorial: Autoimmunity and the Brain: Paraneoplastic Neurological Injury and Beyond. Front. Neurol. 13:900130. doi: 10.3389/fneur.2022.900130

Editorial on the Research Topic

Autoimmunity and the Brain: Paraneoplastic Neurological Injury and Beyond

Autoimmune encephalitides—including the paraneoplastic disorders associated with malignancy—have come to be recognized as major and surprisingly common causes of potentially treatable neurological disease. The disorders, first thought to be rare autoimmune complications of cancer, are now recognized to occur in individuals with and without cancer and to involve antibodies directed against antigens found either at the neuronal cell surface or present in the neuronal cytoplasm or nucleus. Today, we know that conditions associated with antibodies to cell surface antigens may be paraneoplastic or non-paraneoplastic, are usually associated with non-lethal neuronal dysfunction, and are frequently treatable. In contrast, conditions associated with antibodies to intracellular neuronal proteins are usually found in patients with underlying systemic cancer, are characterized by neuronal death, and tend to respond poorly to treatment. At present, over 50 separate antineuronal antibodies have been associated with these disorders, with additional potentially clinically relevant autoantibodies being described every year.

This Research Topic in Frontiers in Neurology addresses several aspects of autoimmune and paraneoplastic encephalitides as understood at this point in time. These include a review of pathogenesis (Greenlee et al.), discussion by Fredrich et al. and Ruiz-Garcia et al. of antibody testing in the diagnosis of these conditions in clinical practice; an intriguing case report by Bartley et al. regarding the use of phage display library in identifying anti-Yo antibody not detectable by immunofluorescence; characterization of antibody-mediated neurological syndromes by Garza and Piquet, Totland et al., and Liu et al.; articles concerning the use of biomarkers in studying these conditions as well as neuromyelitis spectrum disorders by Li Q. et al., Kammeyer et al., Mizenko et al., Chen et al., and Li Y. et al.; and a review by Zoccarato et al. of the important but less-studied area of paraneoplastic neuropathies. Each of these articles makes a valuable contribution the field.

Despite the rapid growth of knowledge in this field, a number of important questions remain to be answered. The first of these, given the steadily expanding number of identified antineuronal autoantibodies, is the urgent need for timely, effective identification of antibodies which at present may require use of multiple testing panels or evaluations. There is also the question of detecting antibodies not yet available in commercial tests or detecting multiple antibodies present in an individual patient. One possible approach, to facilitate early initiation of treatment, could be a uniform system in which laboratories initially reported the presence of antineuronal antibodies, followed by subsequent identification of the antineuronal antibody itself. In this area, the role of the more advanced techniques such as those using phage display analysis remains unstudied; and the report by Bartley et al., which describes phage display detection of anti-Yo antibodies in a case of myelopathy where immunoreactivity to Purkinje cells could not be detected, raises important questions about the use of this technique in diagnosis.

The second of these areas involves pathogenesis, made particularly relevant by the occurrence of autoimmune encephalitides in individuals treated with immune checkpoint inhibitors (Greenlee et al.). Although elegant work by Chefdeville et al. and Small et al. has analyzed expression of NMDAR and CDR2/CDR2L antigens in tumors of patients with anti-NMDAR and anti-Yo antibody responses (1, 2), the sequence of events which leads from tumor expression of antigen to central nervous system involvement has not been delineated. Thus, it is not known why these tumor antigens can provoke a massive immune response at a time when the neoplasm itself is so small as to be undetectable by PET imaging; nor do we actually know the sequence of events which leads to entry of this immune response into the central nervous system and attack on individual neurons. The role of antibodies to neuronal surface proteins such as anti-NMDAR, anti-AMPAR or antibodies to LGI1 and CASPR2 in disease pathogenesis has been extensively studied [Greenlee et al.; (3–5)], and the findings obtained in these studies can almost certainly be extended to other similar autoantibodies. However, the mechanisms of injury identified in studies with these antibodies may or may not be applicable to other antibody-associated syndromes such as the multifocal lesions which occur in $GABA_A$ encephalitis or the central and peripheral manifestations associated with antibodies to CRMP5 [Totland et al.; (6)]. Similarly, although Linnoila et al. have developed an animal model to study the NMDAR encephalitis that can follow herpes simplex virus infection (7), it is unclear why some infections, such as herpes simplex virus encephalitis, generate an antineuronal antibody (anti-NMDAR) response, whereas this is not seen in many other infectious and non-infectious processes causing neuronal death.

In contrast to our knowledge concerning antibodies to neuronal surface membrane antigens, the role of antibodies to intracellular neuronal antigens—such as anti-Yo or anti-Hu—remains controversial. Although both anti-Yo and anti-Hu antibodies from affected patients have been shown to be taken up by neurons *in vitro* and to produce neuronal death (8, 9), antibody-mediated neuronal injury has not been proven in an animal model. It should be noted, however, that the great majority of attempts to develop an animal model have studied anti-Yo antibodies, and that virtually all of these experiments have immunized animals with proteins or DNA encoding the Yo antigen, CDR2 (Greenlee et al.). This is important because recent work strongly suggests that the pathogenic antigen involved in anti-Yo antibody response is not CDR2, as has long been assumed, but, rather, the related protein, CDR2L (10); and attempts to produce an animal model using CDR2L have not been reported. Similar concerns exist concerning the role of T lymphocytes in pathogenesis. Although brains of affected patients with anti-Yo or anti-Hu antibodies often contain infiltrates of cytotoxic T lymphocytes, neuronal injury by sensitized T lymphocytes duplicating paraneoplastic disease has also never been demonstrated *in vitro* or in an animal model (Greenlee et al.). An unanswered—and significant—question is how T lymphocytes might target neurons, given that normal adult neurons do not express the MHC class I molecules needed to allow recognition by cytotoxic T cells (11). Neuronal upregulation of MHC class I expression has been described in other clinical and experimental settings (12, 13), but this has not been analyzed in human paraneoplastic neurological disease or in neurons exposed to anti-Yo or anti-Hu antibodies *in vitro* or *in vivo*. A major roadblock to our understanding of disease pathogenesis thus remains the lack of animal models which parallel the natural course of human paraneoplastic and other autoimmune encephalitides seen in humans.

An additional area where our knowledge is inadequate has to do with the pathogenesis of the various categories of peripheral nerve injury associated with cancer. Subacute sensory neuronopathy was the prototypical paraneoplastic disorder affecting the peripheral nervous system (e.g., anti-Hu and anti-CRMP5). The spectrum of immune mediated neuropathies has greatly expanded over the past decade and now includes neuronal surface antibodies such as CASPR2 (14). Some antibody-associated disorder have both central and peripheral symptoms such as anti-KLHL11 and PCA2 antibodies, further expanding the phenotype of peripheral nervous system disorders (Zoccarato et al.). Additionally, neuropathies associated with antibody to myelin-associated glycoprotein and their role in neuropathies associated with plasma cell dyscrasias need to be further investigated [Zoccarato et al.; (15, 16)].

The final—and clinically most important—area has to do with treatment of affected patients. Although several authors such as Graus et al. and Abboud et al. have published excellent clinical guidelines for provisionally diagnosing these disorders and initiating immunomodulatory therapies (16, 17), treatment of these disorders remain empiric, without controlled trials to guide the use of modalities including corticosteroids, immunoglobulin G (IgG), plasma exchange, or agents such as rituximab. In this regard, the first international, multi-institutional, double-blind NIH NeuroNext trial (NN111, ExTINGUISH Trial, ClinicalTrials.gov: NCT04372615) involving the CD19-specific monoclonal, inebilizumab, in NMDAR encephalitis represents an exciting and important step forward. Badly needed—for patients with antibodies to neuronal surface antigens as well as patients with antibodies to intracellular neuronal antigens—are

actual studies, using standardized protocols involving the agents currently in use, such as corticosteroids, IVIG, PLEX, or rituximab singly or in combination. Such studies would be difficult to fund but could conceivably be carried out over time on a less formal multi-institutional basis and, like use of pre-clinical animal models, could provide invaluable information regarding treatment. Future studies also need to explore and validate more robust clinical outcomes measures beyond the modified Rankin scale, which is heavily weighted toward motor deficits and does not encompass cognitive impairment and psychiatric/behavioral sequelae seen in frequently seen in patients with autoimmune encephalitis. These future scoring systems could even help identify patients who may benefit from more aggressive/longer duration immunotherapy or more meaningful outcomes such as resumption of gainful employment or schooling.

The decade ahead promises to be fascinating in terms of advancement of knowledge and development of new diagnostic and therapeutic approaches for this important group of disorders.

AUTHOR CONTRIBUTIONS

JG, NC, SC, and CV conceived and wrote the initial draft of this manuscript. JA and IH contributed to the final revision as submitted. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a Merit Review Award from the United States Department of Veterans Affairs (JG) awards from the Western Institute for Biomedical Research (JG and SC) and by grants from Helse Vest (CV).

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frontiers in Immunology

ORIGINAL RESEARCH
published: 16 June 2021
doi: 10.3389/fimmu.2021.680424

High Level of Soluble CD146 In Cerebrospinal Fluid Might be a Biomarker of Severity of Anti-N-Methyl-D-Aspartate Receptor Encephalitis

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Background: Disruption of the blood–brain barrier (BBB) is an important pathophysiological process of anti-N-methyl-D-aspartate receptor (anti-NMDAR) encephalitis. A recent multi-center study showed that soluble (s) CD146 is a potential biomarker for monitoring early BBB damage and central nervous system inflammation, but little is known about sCD146 in anti-NMDAR encephalitis.

Method: Twenty-three anti-NMDAR encephalitis patients and seventeen controls with non-inflammatory neurological diseases were recruited. sCD146 and inflammatory cytokines in cerebrospinal fluid (CSF) and serum were detected by ELISA. Modified Rankin scale (mRS) scores were used to assess the neurological status of each patient. A follow-up review was completed three months after discharge.

Results: sCD146 levels in the CSF of patients with the acute stage anti-NMDAR encephalitis were significantly increased compared with controls and accompanied by increases in TNF-α, IL-6 and IL-10. CSF sCD146 was positively correlated with neuroinflammatory factors in the CSF and with mRS score. Three months after effective treatment, CSF sCD146 in patients was significantly decreased but remained significantly different compared with the controls.

Conclusion: Our data suggested that higher expression of CSF sCD146 correlated with more serious neurological damage. Therefore, levels of CSF sCD146 may represent a promising indicator for monitoring disease and optimizing clinical treatment decisions in the early stages of anti-NMDAR encephalitis.

Keywords: anti-NMDAR encephalitis, soluble CD146, cerebrospinal fluid, blood–brain barrier, neurological damages

OPEN ACCESS

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

Received: *14 March 2021*
Accepted: *26 May 2021*
Published: *16 June 2021*

Citation:
Li Q, Chen J, Yin M, Zhao J, Lu F, Wang Z, Yu X, Wang S, Zheng D and Wang H (2021) High Level of Soluble CD146 In Cerebrospinal Fluid Might be a Biomarker of Severity of Anti-N-Methyl-D-Aspartate Receptor Encephalitis. Front. Immunol. 12:680424. doi: 10.3389/fimmu.2021.680424

INTRODUCTION

Anti-N-methyl-D-aspartate receptor (anti-NMDAR) encephalitis has gradually become recognized as an autoimmune disease targeting neuronal synapses. Generally, neuropsychiatric symptoms, including cognitive dysfunction, seizure, abnormal movements, autonomic instability and hypoventilation, are the most classic clinical features to be presented (1–4). Although anti-NMDAR antibodies can be found in the serum and cerebrospinal fluid (CSF) of patients with anti-NMDAR encephalitis, disruption of the blood–brain barrier (BBB) in anti-NMDAR encephalitis remains unclear.

CD146 is an adhesion molecule which was discovered in 1987 in the plasma membrane of human melanoma cells (5, 6). In recent decades, CD146 has been shown to be a type of transmembrane glycoprotein primarily expressed at the intercellular junctions of endothelial cells in the blood vessels of the central nervous system (CNS), and plays a key role in controlling the permeability and integrity of vessels (5–7). The soluble form of CD146 (sCD146) comes from shedding of the extracellular portion of CD146 via matrix metalloproteinases (MMPs) (5–7). Studies have proven that MMPs are elevated in anti-NMDAR encephalitis (8, 9), and to date, higher sCD146 has been found in chronic obstructive pulmonary disease (10), active inflammatory bowel disease (11), systemic sclerosis (12, 13), chronic renal disease (14, 15) and CNS diseases including multiple sclerosis (MS) (16, 17), neuromyelitis optica (16), CNS infections, peripheral neuropathy and Alzheimer's disease (4–6, 18, 19). BBB integrity and low permeability are necessary for maintaining normal function of the CNS, and BBB disruption may play an important role in anti-NMDAR encephalitis (16, 18, 20). Thus, based on a recent multi-center study in MS with large samples conducted by Wang et al. (16), the results showed that compared with other soluble adhesion molecules, sCD146 may become a potential biomarker as it has high sensitivity and specificity for evaluating early BBB damage and CNS inflammation. Currently, little is known about sCD146 in anti-NMDAR encephalitis. In this study, we measured the levels of sCD146 in the CSF and serum of patients with anti-NMDAR encephalitis and assessed its potential clinical value in the diagnosis and treatment of this disease.

MATERIALS AND METHODS

Patients and Controls

In this study, twenty-three patients who were diagnosed with anti-NMDAR encephalitis from 2018 to 2020 according to the revised anti-NMDAR encephalitis diagnosis criteria of 2016 were recruited from the Department of Neurology, The Affiliated Brain Hospital of Guangzhou Medical University, Guangzhou, China. The diagnosis of anti-NMDAR encephalitis was confirmed based on clinical manifestations and positive identification of antibodies against the NRI subunit of NMDAR in the CSF by cell-based analysis. Seventeen patients with non-inflammatory neurological diseases were selected as controls, including seven cases of Parkinson's disease and ten cases of normal pressure hydrocephalus. Both anti-NMDAR encephalitis patients and controls were negative for the detection of common viruses and other pathogens by PCR. The study was approved by the Ethics Committee of The Affiliated Brain Hospital of Guangzhou Medical University. All participants provided informed consent before proceeding with the subsequent study. CSF samples from patients and controls were collected by lumbar puncture within three days of admission for diagnosis; blood samples were collected at the same time. CSF and blood samples were processed within 30 min of collection and centrifuged at 4000 rpm for 5 min. Supernatants of the CSF and serum samples were separated as soon as possible, transferred, numbered and stored at −80°C until they were used in ELISAs. The integrity of the BBB was evaluated by calculating the CSF to serum albumin quotient (QAlb), which represented an approximation of BBB breakdown. Modified Rankin Scale (mRS) scores were used to assess the neurological status of each patient. In addition to tumor removal, all patients were treated with first-line immunotherapy comprising intravenous methylprednisolone (1000 mg for 3–5 days), intravenous immunoglobulin (IVIg) (1 g/kg for 5 days per cycle) or plasma exchange, alone or combined (21, 22). Three months after discharge, patients were followed up and CSF and serum samples were obtained, along with reassessment by mRS.

Detection of sCD146 by ELISA

Commercial sandwich ELISA kits were used to detect the inflammatory cytokines tumor necrosis factor-α (TNF-α) (Cusabio, Wuhan, China), matrix metalloproteinase-2 (MMP-2), interleukin-6 (IL-6), IL-10 and sCD146 (Bender Med-Systems GmbH Campus, Vienna, Austria) in the CSF and serum. Assays were performed according to the manufacturer's instructions.

Statistical Analysis

Descriptive statistics were performed on demographic factors and clinical features of patients. The Kruskal-Wallis test was used to analyze the differences in TNF-α, MMP-2, IL-6, IL-10, and sCD146 levels among subgroups. Pearson's test or Spearman's test were used to evaluate the correlations between TNF-α, MMP-2, IL-6, IL-10, sCD146 and mRS scores. A value of $P<0.05$ was considered statistically significant, and all statistical analyses were conducted using SPSS version 17.0 (IBM Corp, Armonk, NY).

RESULTS

Patient Characteristics

Detailed clinical features and levels of inflammatory cytokines in CSF or serum collected from patients with anti-NMDAR encephalitis (n=23) and controls with non-inflammatory neurological diseases (n=17) are presented in **Table 1**. Only two anti-NMDAR patients (8.7%) had a history of teratomas. Psychiatric symptoms (82.6%) was the most common clinical presentation. All patients had moderate to severe disability with mRS from 3–5. Ten patients (43.78%) had high QAlb values. The degree of BBB damage was calculated according to $QAlb\times10^3$, where values <6 indicate no BBB damage; 6–8, mild; 8.1–10, moderate; >10, severe damage. Mild dysfunction was detected in

TABLE 1 | Clinic characteristics of anti-NMDAR encephalitis patients and controls.

	Anti-NMDAR Encephalitis (n = 23)	Control (n = 17)	p value
Gender (male/female)	8/15	8/9	0.000
Age (years, mean ± SD)	29.6±14.2	27.5±9.9	0.208
Clinic symptoms (n, %)			
Fever	1 (4.4)	0	"-
Psychiatric symptom	19 (82.6)	0	"-
Disorders of memory	2 (8.7)	0	"-
Seizure	12 (52.2)	0	"-
Abnormal movements	1 (4.4)	0	"-
Automatic instability	3 (13.1)	0	"-
Hypoventilation	0 (0)	0	"-
CSF routine (mean ± SD)			
WBC (×10E6/L)	7.8±25.7	2.0±2.2	0.000
GLU (mmol/l)	3.7±0.6	3.7±0.6	0.924
Cl (mmol/L)	116.5±6.5	125±3.4	0.000
Treatment (n, %)			
Plasma exchange	5 (21.7)	"-	"-
IVIg	16 (69.6)	"-	"-
Steroids	18 (78.3)	"-	"-
Qalb (mean ± SD, damage %)	6.4±3.8 (43.5)	4.445±1.167 (11.8)	0.001
CSF anti-NMDAR antibody positive (n, %)	23 (100)	0	"-
Tumor comorbidity (n, %)	2 (8.7)	0	"-
Max mRS			
3 (n, %)	8 (34.8)	"-	"-
4 (n, %)	10 (43.5)	"-	"-
5 (n, %)	5 (21.7)	"-	"-

four patients, moderate in two and severe in four. IVIg (69.57%) and steroids (78.26%) were the major treatments.

sCD146 Was Increased in the CSF of Anti-NMDAR Encephalitis in the Acute Stage and Decreased After Three Months of Treatment

The expression of sCD146 in CSF of patients was notably increased in anti-NMDAR encephalitis patients in the acute stage compared with controls (P<0.001) but no difference was shown in serum sCD146 between these two groups. The QAlb and CSF MMP2 of anti-NMDAR patients were also significantly higher than those in the control group, as were the levels of CSF TNF-α, IL-10, and IL-6 (P<0.001).

All patients were followed up for three months after receiving different treatments. CSF sCD146 levels were significantly decreased from 54.0±32.2 to 29.9±8.5 (ng/ml) (P=0.001), and TNF-α, IL-10 and IL-6 in CSF were also decreased significantly (**Table 2**). Despite this, both sCD146 and inflammatory factors remained significantly elevated in anti-NMDAR patients compared with controls (**Figure 1**). However, neither serum nor CSF levels of MMP2 in the follow-up differed from levels detected during the acute stage. The mRS of all patients dropped below 3 at follow-up.

CSF sCD146 Was Positively Correlated With Clinical Parameters of Anti-NMDAR Encephalitis Patients in the Acute Stage, Including Multiple Inflammatory Factors and mRS

As shown in **Figure 2**, significant positive correlations were found between sCD146 levels and IL-10 (r=0.479, p=0.021), IL-6 (r=0.452, p=0.031), and MMP2 (r=0.415, p=0.049) in the CSF of anti-NMDAR encephalitis patients in the acute stage. No significant correlations were shown between CSF sCD146 and inflammatory factors in patients at the 3-month follow-up or in the non-inflammatory disease control group.

There was a significant positive correlation between CSF sCD146 levels and mRS scores at the acute stage (r=0.417,

TABLE 2 | Expression of inflammatory factors in the CSF and serum of anti-NMDAR encephalitis patients at the acute stage and 3-month follow up.

	Acute stage	Follow up	p value
TNF-α (pg/ml)	7.7±5.0	4.4±2.9	0.000
IL-10 (pg/ml)	6.9±3.4	3.5±1.7	0.000
IL-6 (pg/ml)	10.5±5.1	6.0±1.6	0.000
Serum MMP2 (ng/ml)	241.3±90.8	232.6±84.7	0.621
CSF MMP2 (ng/ml)	36.6±15.0	31.9±13.1	0.170
Serum sCD146 (ng/ml)	501.8±124.5	488.0±102.2	0.396
CSF sCD146 (ng/ml)	54.0±32.2	29.9±8.5	0.001
mRS	3.9±0.8	1.8±1.1	0.000

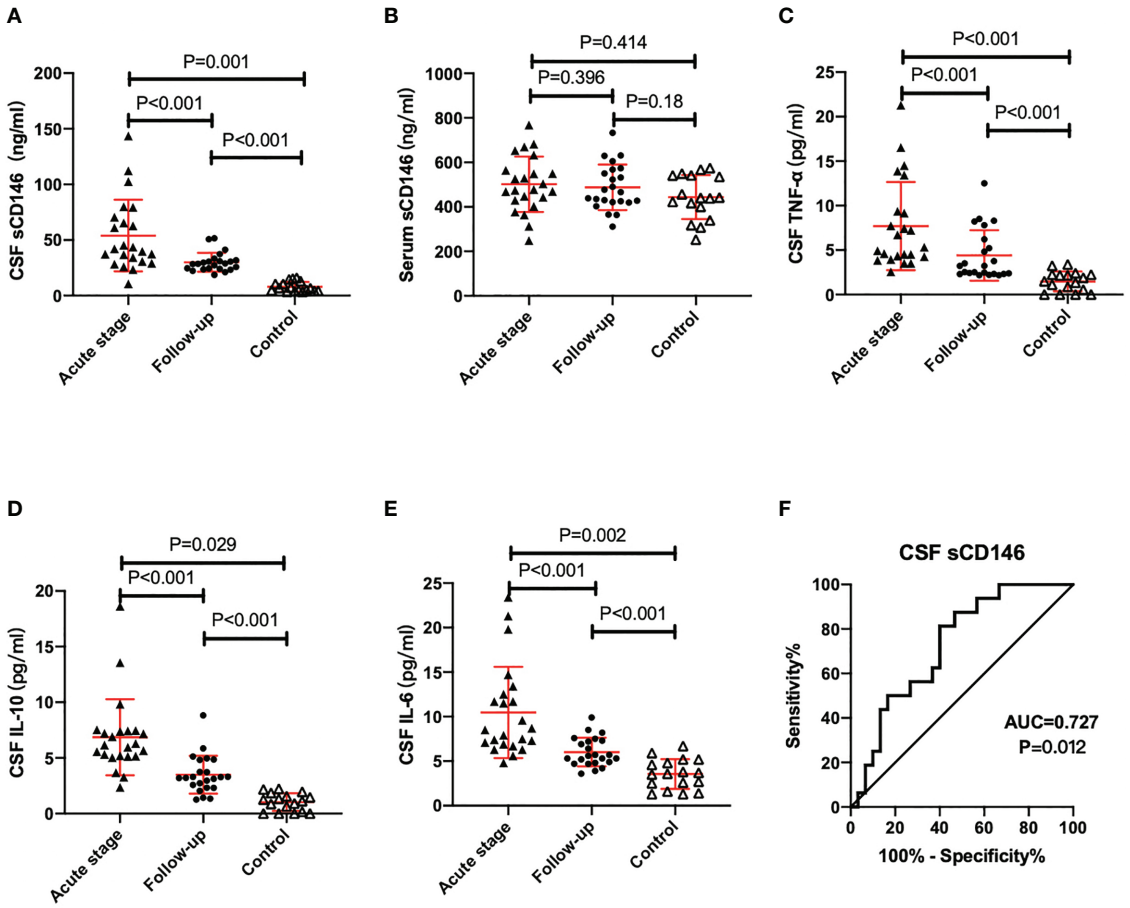

FIGURE 1 | sCD146 levels in the CSF of patients with acute stage anti-NMDAR encephalitis were significantly increased compared with controls and 3-month follow-up accompanied by increases in TNF-α, IL-6 and IL-10 **(A–E)**. The ROC curve of CSF sCD146 to predict the severity of neurologic impairments in anti-NMARD encephalitis patients **(F)**.

p=0.004) (**Figure 2D**). To evaluate the ability of CSF sCD146 to indicate the severity of neurologic impairments in patients with anti-NMDAR encephalitis, we further performed receiver operating characteristic curve (ROC) analysis (**Figure 1F**). Here we defined the severity according to mRS scores (3, moderate disability; >3, severe disability). The area under curve was 0.727 (p=0.012; cut-off value, 33.75 ng/ml; Youden index, 0.418)

DISCUSSION

In our study, CSF and serum of twenty-two anti-NMDAR encephalitis patients and seventeen controls with non-inflammatory neurological diseases were examined. We found that sCD146 levels were markedly elevated in the CSF of anti-NMDAR encephalitis patients. Moreover, this biomarker was significantly associated with disease severity. To our knowledge,

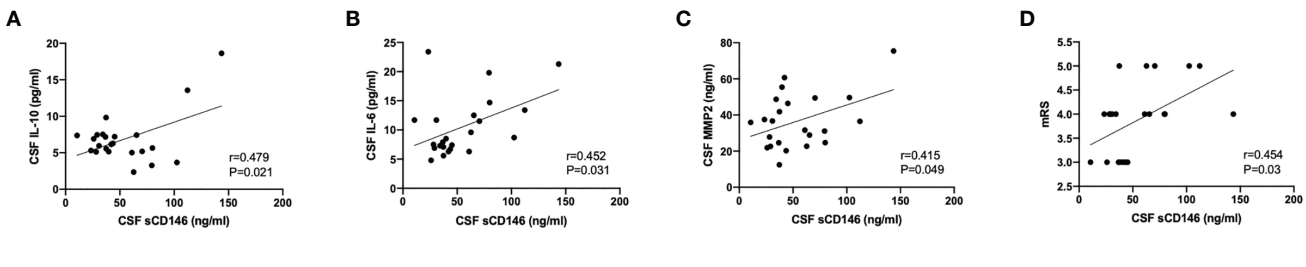

FIGURE 2 | CSF sCD146 was positively correlated with neuroinflammatory factors in the CSF and with mRS score **(A–D)**.

this study is the first to report changes in sCD146 levels in the CSF of patients suffering from this disease.

Disruption of the BBB is present in a variety of neuroinflammatory diseases. In a previous study of in vitro BBB and mouse models, Chen et al. (7) reported that CD146 was expressed universally in different regions of the brain and played a vital role in the formation and function of the BBB. In a recent study, Wang et al. (16) showed that CSF sCD146 directly promoted BBB hyperpermeability in active MS. sCD146 was found not only to be a sensitive marker for BBB damage but also a novel driver of neuroinflammatory dysfunction (5, 16, 23). Therefore, we speculated that there was significant BBB dysfunction in patients with acute anti-NMDAR encephalitis. Endothelial cells of the BBB are more easily damaged under this inflammatory state while the upregulation of sCD146 can impair the permeability of the BBB and thus aggravate disease severity.

QAlb is often used as a common indicator to evaluate destruction of the BBB. In this study, we found that the mean QAlb of anti-NMDAR encephalitis patients was abnormally high compared with controls, suggesting impairment of the BBB. Despite QAlb being elevated in 43.48% (10/23) of patients, no significant correlation was found between QAlb and CSF sCD146. Similar to our data, Wu, et al. found that only 15.21% of patients in the acute stage had elevated QAlb (20). Previous researches showed that there were several factors that might influence albumin levels in the CSF including age, rate of CSF transport or production, and supraspinal lesions, among others (24, 25). Therefore, QAlb might be an imprecise way to assess BBB dysfunction (16, 17, 24, 26), especially in patients with anti-NMDAR encephalitis. The value of QAlb remains worthy of further study.

In this study, we found CSF IL-6, TNF-α and IL-10 to be increased in anti-NMDAR encephalitis patients in the acute stage. We further investigated the relationship between sCD146 and the proinflammatory cytokines IL-6 and TNF-α, and the anti-inflammatory cytokine IL-10. The results showed that all these three proinflammatory factors were positively correlated with CSF sCD146. In previous studies, abnormal elevation of sCD146 has been reported to correlate with proinflammatory factors, such as TNF-α, IL-13 and IL-17 (5, 27). Early research by our team confirmed that the presence of inflammatory factors in the CSF of anti-NMDAR encephalitis patients reflect the activity of the disease (8, 9, 17, 28–30). In the present study, we considered that local inflammation in the brain was activated, leading to the accumulation of inflammatory factors in the CSF that enhanced the expression of sCD146, ultimately promoting BBB dysfunction. Our results reflected the disturbances in vascular physiology and indicated that the co-activation of these factors might play an important role in the pathogenesis of anti-NMDAR encephalitis. Additional studies are needed to confirm that.

Three months after effective treatment, the levels of sCD146 in CSF of the patients were significantly decreased. Eleven patients (47.8%) recovered well (mRS=1), but seven patients (30.43%) remained with mRS scores above 3 and none had an mRS score of 0, which meant that no patients had achieved a complete recovery in 3 months. Similar research by Titulaer et al. (22) showed that only 53% of patients had symptom improvement within four weeks of first-line immunotherapy. We hypothesized that although inflammation of the brain was partially improved after treatment, BBB dysfunction persisted for a long time because there was long-term high expression of CSF sCD146 and other inflammatory factors. Continued inflammation in the CNS was consistent with the clinical features of slow recovery from anti-NMDAR encephalitis (4).

Here we have shown that the level of CSF sCD146 was positively associated with mRS in the acute stage of anti-NMDAR encephalitis. ROC curve analysis showed that CSF sCD146 ≥33.75 ng/ml can be used as an index to predict the severity of anti-NMDAR encephalitis, and the accuracy of CSF sCD146 in indicating the severity of this disease was remarkable. From this we could infer that the higher the expression of CSF sCD146, the more serious the neurological damage. Our findings implied that CSF sCD146 might hold potential in assessing the severity of anti-NMDAR encephalitis.

Since the initial description of this disease in 2007 (19), the optimal treatment of anti-NMDAR encephalitis has not yet been clarified (21). Previous studies showed that 19.4%–25.5% of anti-NMDAR encephalitis patients remained refractory to current immunotherapies and a significant number of those patients suffered prolonged neurological dysfunctions (21, 22, 31). Since early treatment is closely linked to a better prognosis for patients (22, 32), our study indicates that the short-term immunotherapies we routinely used were effective but may not be comprehensive enough, especially for those patients with persistently high expression of CSF sCD146. It was necessary to strengthen the treatment continuously to relieve the inflammation and facilitate recovery; an early combination with second-line therapy, even novel immunotherapies became particularly important. Therefore, the levels of CSF sCD146 may become a promising indicator for disease monitoring and optimizing clinical treatment decisions in the early stage in anti-NMDAR encephalitis.

Furthermore, it was reported in MS that CSF sCD146 could enhance the expression of adhesion molecules and promote the migration of leukocytes into the CNS (17). Our previous study showed the levels of sICAM-1, sVCAM-1 and sL-selectin in CSF were significantly elevated in anti-NMDAR encephalitis patients. Combined with our findings (30), it may share a similar pathophysiological mechanism in anti-NMDAR encephalitis. We hope to further explore this in our future research.

There were several limitations to our study. First, this was a single-center study, the nature of which may have introduced biases related to a small sample size and a shorter follow-up period. Second, we analyzed CSF and serum samples from anti-NMDAR encephalitis patients but did not conduct further study on pathogenesis.

CONCLUSION

In this study, we found that the CSF sCD146 was significant increased in anti-NMDAR encephalitis patients with the acute stage, which were positively correlated with the levels of neuroinflammatory factors and mRS score. These findings suggested that the higher the expression of CSF sCD146, the more severe the neurological damage. Moreover, long-term

high expression of CSF sCD146 and other inflammatory factors was accompanied by BBB dysfunction. Therefore, the levels of CSF sCD146 may represent a promising indicator for optimizing clinical treatment decisions in the early stage and disease monitoring in anti-NMDAR encephalitis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved by the Ethics Committee of The Affiliated Brain Hospital of Guangzhou Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was supported by Guangzhou Planned Project of Science and Technology (202102080073) and Natural Science Foundation of Guangdong Province (2019A1515011434, 2019A1515011611).

ACKNOWLEDGMENTS

We thank Gillian Campbell, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

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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.

frontiers in Immunology

BRIEF RESEARCH REPORT
published: 29 June 2021
doi: 10.3389/fimmu.2021.691536

Limitations of a Commercial Assay as Diagnostic Test of Autoimmune Encephalitis

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OPEN ACCESS

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

Received: 06 April 2021
Accepted: 15 June 2021
Published: 29 June 2021

Citation:
Ruiz-García R, Muñoz-Sánchez G, Naranjo L, Guasp M, Sabater L, Saiz A, Dalmau J, Graus F and Martinez-Hernandez E (2021) Limitations of a Commercial Assay as Diagnostic Test of Autoimmune Encephalitis. Front. Immunol. 12:691536. doi: 10.3389/fimmu.2021.691536

Detection of neuronal surface antibodies (NSAb) is important for the diagnosis of autoimmune encephalitis (AE). Although most clinical laboratories use a commercial diagnostic kit (Euroimmun, Lübeck, Germany) based on indirect immunofluorescence on transfected cells (IIFA), clinical experience suggests diagnostic limitations. Here, we assessed the performance of the commercial IIFA in serum and CSF samples of patients with suspected AE previously examined by rat brain immunohistochemistry (Cohort A). Of 6213 samples, 404 (6.5%) showed brain immunostaining suggestive of NSAb: 163 (40%) were positive by commercial IIFA and 241 (60%) were negative. When these 241 samples were re-assessed with in-house IIFA, 42 (18%) were positive: 21 (9%) had NSAb against antigens not included in the commercial IIFA and the other 21 (9%) had NSAb against antigens included in the commercial kit (false negative results). False negative results occurred more frequently with CSF (29% vs 10% in serum) and predominantly affected $GABA_BR$ (39%), LGI1 (17%) and AMPAR (11%) antibodies. Results were reproduced in a separate cohort (B) of 54 AE patients with LGI1, $GABA_BR$ or AMPAR antibodies in CSF which were missed in 30% by commercial IIFA. Patients with discordant $GABA_BR$ antibody results (positive in-house but negative commercial IIFA) were less likely to develop full-blown clinical syndrome; no significant clinical differences were noted for the other antibodies. Overall, NSAb testing by commercial IIFA led to false negative results in a substantial number of patients, mainly those affected by anti-LG1, $GABA_BR$ or AMPAR encephalitis. If these disorders are suspected and commercial IIFA is negative, more comprehensive antibody studies are recommended.

Keywords: neuronal antibodies, brain immunohistochemistry, diagnostic test, autoimmune encephalitis (AE), immunofluorescent assay

INTRODUCTION

Detection of antibodies to neuronal surface proteins and synaptic receptors is important to establish a definitive diagnosis of autoimmune encephalitis (1). Well-characterized clinical syndromes associate with specific antibodies, and previously unrecognized neurological diseases are currently defined by the corresponding neuronal surface antibody, such as anti-NMDAR encephalitis or anti-LGI1 encephalitis, which are the most frequent antibody-mediated encephalitis (2). Commercial diagnostic kits using transfected cells that express the most common neuronal surface antigens are widely available, and allow rapid antibody testing in clinical laboratories (3). Most clinical laboratories worldwide use the same commercial indirect immunofluorescence assay (IIFA) whereas rat brain immunohistochemistry is only performed in a few specialized centers (4). However, there are no studies comparing the performance of commercial IIFA with the combination of rodent brain immunohistochemistry and IIFA as used in the initial description of most neuronal surface antibodies. There is preliminary data suggesting that the sensitivity of commercial IIFA, particularly for LGI antibodies in cerebrospinal fluid (CSF), may be low, and false positive results may occur particularly when serum is used at high concentration (4, 5). Moreover, commercial diagnostic kits contain a limited number of antigens (up to 6 specificities) and some less frequent or recently described antigens are not included. For these reasons, the use of commercial IIFA as the only method to diagnose autoimmune encephalitis probably misses the detection of otherwise well-characterized and relevant antibodies. This can have important implications such as overlooking the presence of tumors typically associated with some of the non-detected antibodies, or not giving immunotherapy to patients with unrecognized autoimmune encephalitis. Here, we assessed the diagnostic value and limitations of a commercial kit for the detection of neuronal surface antibodies in the serum and CSF of patients with autoimmune encephalitis.

PATIENTS AND METHODS

Patients and Samples

We prospectively examined 6213 serum and CSF samples from patients referred to our diagnostic lab for detection of antibodies against neuronal surface antigens from October-2016 to October-2020 (Cohort A). Samples were screened with rat brain immunohistochemistry and results were examined by two independent observers. Samples showing positive immunostaining suggestive of a neuropil antibody were first studied with commercial IIFA (6). Samples that were positive on brain immunohistochemistry (with a pattern of staining suggesting a neuronal surface antibody) but negative on commercial IIFA were later studied with in-house IIFA. Clinical data was reviewed in all cases with available information. To further assess the performance of the commercial IIFA, we retrospectively studied 54 consecutive CSF samples from patients with encephalitis and LGI1 (n=12), AMPAR (n=19) or $GABA_BR$ (n=23) antibodies confirmed by brain immunohistochemistry and in-house IIFA (Cohort B).

Rat Brain Immunohistochemistry

Tissue immunohistochemistry was performed as previously described (7). Briefly, adult Wistar rats were euthanized in a CO_2 chamber and the brain was removed without previous tissue perfusion. Brains were sagittally split in two hemispheres, immersed in 4% paraformaldehyde for 1h at 4°C, cryoprotected with 40% sucrose for 48h, and snap frozen in chilled isopentane. Frozen sections were air-dried for 30 min and sequentially treated with hydrogen peroxide 30% in PBS for 15 minutes. Brain sections were blocked with 5% normal goat serum in PBS for 1h at room temperature and incubated with patients' sera (diluted 1:200) or CSF (1:2) overnight at 4°C. Biotinylated goat anti-human IgG (Vector Labs, Burlingame, CA, 114 USA) was added for 2 h, followed by incubation with the avidin–biotin immunoperoxidase complex (Vector Labs, Burlingame, CA, 114 USA) for 1 h. The reaction was developed with 0.05% diaminobenzidine (Sigma, St. Louis, MO, USA).

Indirect Immunofluorescence Assays

Samples that produced a neuropil immunostaining on rat brain immunohistochemistry were subsequently examined with two types of IIFAs: 1) the Autoimmune Encephalitis Mosaic 6 kit (Euroimmun, Lübeck Germany), following manufacturer's instructions and recommended dilutions (undiluted CSF and 1:10 serum), to test IgG antibodies against N-methyl-D-aspartate (NMDA) receptor (GluN1), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor (GluA1, GluA2), gamma-aminobutyric (GABA) B receptor (B1 and B2 subunits), contactin-associated protein-like 2 (CASPR2), leucine-rich glioma-inactivated protein 1 (LGI1) and dipeptidyl-peptidase 6 (DPPX), 2) in-house IIFAs in which HEK293 cells were transfected with DNA constructs to express the following antigens: NMDA receptor (GluN1, GluN2), AMPA receptor (GluA1, GluA2), $GABA_B$ receptor (B1, B2), CASPR2, LGI1 (with and without disintegrin and metalloproteinase domain-containing protein 23 [ADAM23] co-transfection), $GABA_A$ receptor (α1, β3), metabotropic glutamate receptors mGluR1, mGluR2, mGluR5, Ig-Like Domain-Containing Protein 5 (IgLON5) or Seizure 6-like protein 2 (SEZ6L2) as previously described (7–17). Briefly, sera and CSF were diluted in PBS-1% BSA (1:2 CSF and 1:40 serum) and incubated with stored pre-fixed transfected cells overnight, and with an anti-human IgG antibody conjugated with AF488 or AF594 (Invitrogen) for 1 hour. Live, non-fixed transfected cells were used for detection of GPI-LGI1 (without ADAM23), $GABA_A$ receptor, mGluR1-2-5, IgLON5 and SEZ6L2 antibodies. IIFA results were observed in an Axio-Imager 2 microscope (Zeiss, Germany).

The study was approved by the ethics committee of Hospital Clínic of Barcelona. Patients' samples were coded and clinical information was anonymized prior to analysis. Written inform consent was not required as the study was observational, and the

detection of neuronal surface antibodies was requested as part of the routine diagnostic work-up.

RESULTS

In Cohort A, 404 (6.5%) of the 6213 samples (222 sera, 182 CSF) showed a positive staining on brain immunohistochemistry suggesting the presence of neuronal surface antibodies. These 404 samples were analyzed by the commercial IIFA and 163 (40%) resulted positive for IgG against one of the included antigens: 68 (42%) for NMDAR [27 sera/41 CSF], 52 (32%) for LGI1 [26 sera/26 CSF], 16 (10%) for AMPAR [11 sera/5 CSF], 15 (9%) for CASPR2 [9 sera/6 CSF], 11 (7%) for $GABA_BR$ [6 sera/5 CSF] and 1 (1%) for DPPX [serum] antibodies (**Figure 1**). Samples with positive brain immunohistochemistry and negative commercial IIFA results (241 samples) were further analyzed for neuronal surface antibodies by in-house IIFA. We performed the most suitable antigen specific assay depending on the immunostaining pattern and/or the clinical phenotype. Twenty-one (9%) of these 241 samples were positive for antibodies against antigens not included in the commercial kit (13 IgLON5, 3 SEZ6L2, 2 mGluR1, 1 mGluR2, 1 mGluR5, and 1 $GABA_AR$) (**Figure 1**). Additionally, 21 (9%) of the 241 commercial IIFA-negative samples showed a positive result on the in-house IIFA for antigens included in the commercial kit: 11 LGI1 (4 sera/7 CSF), 7 $GABA_BR$ (1 serum/6 CSF), 2 AMPAR (2 CSF), and 1 NMDAR (serum) (**Figures 1** and **2**). These 21 samples were considered as false negative results of the commercial IIFA. The frequency of false-negatives was 39% (7/18) for $GABA_BR$, 17% (11/63) for LGI1, 11% (2/18) for AMPAR, and 1.4% (1/69) for NMDAR antibodies. Among 41 patients with paired serum and CSF samples we obtained false negative results in 7 (17%): antibodies were not detected in one sample by the commercial IIFA in 5/15 patients with LGI (3 in serum, 2 in CSF) and 1/5 with AMPAR antibodies (in CSF), and not detected in serum and CSF in 1/3 patients with $GABA_BR$ antibodies (**Supplementary Table**). The commercial kit failed to detect $GABA_BR$, LGI1 and AMPAR antibodies more frequently in CSF than serum (29% [15/51] of CSF samples compared to 10% [5/48], respectively, p=0.024).

Considering that CSF samples gave more discordant results between commercial and in-house IIFAs and CSF antibody detection is crucial for the diagnosis of autoimmune encephalitis, we assessed 54 additional CSF samples of patients with encephalitis and $GABA_BR$, LGI1 or AMPAR antibodies, confirmed by brain immunohistochemistry and in-house IIFA, and retested them by commercial IIFA (Cohort B). The commercial kit failed to detect antibodies in 16 (30%) samples: 4/12 (33%) with LGI1, 7/23 (30%) with $GABA_BR$, and 5/19

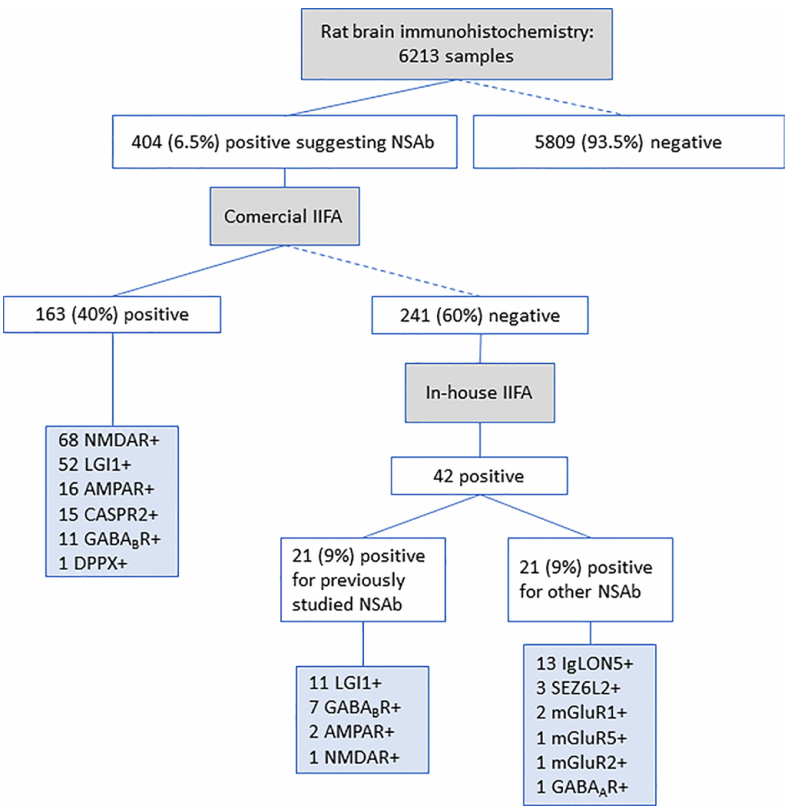

FIGURE 1 | Antibody detection in patients from Cohort A. Workflow used to identify IgG neuronal surface antibodies in a cohort of 6231 samples. IIFA, Indirect immunofluorescent assay; NSAb, Neuronal surface antibodies.

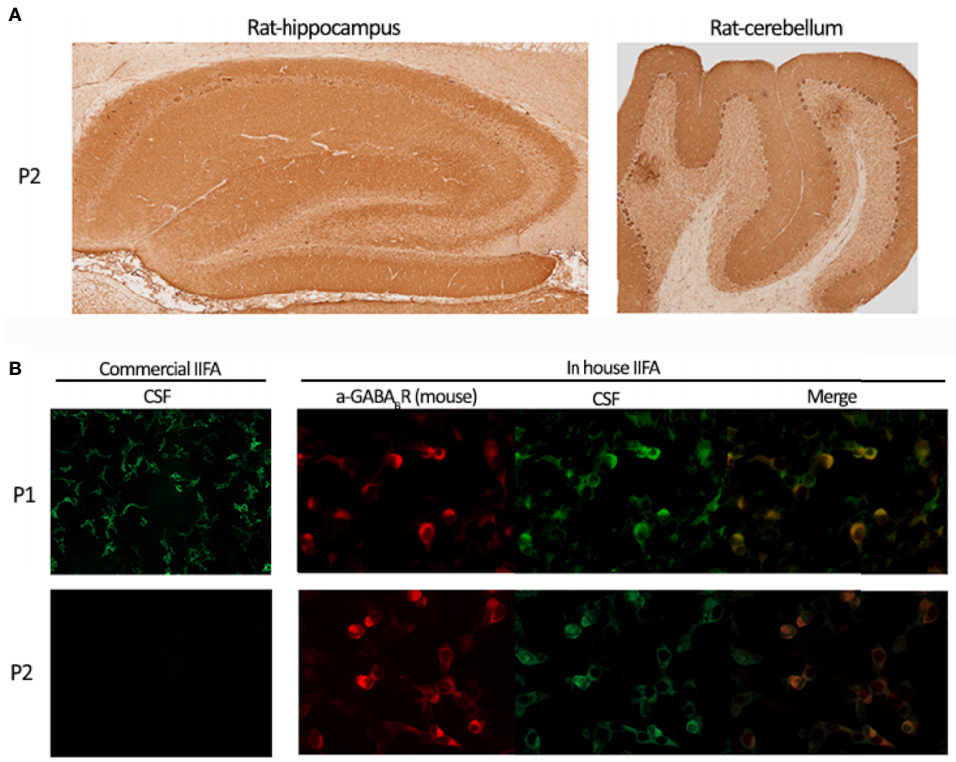

FIGURE 2 | Discrepancies identifying GABA$_B$R antibodies by IIFA. **(A)** One of the two patients' CSF (P2) demonstrating GABA$_B$R immunoreactivity on rat brain immunohistochemistry. Hippocampus (left panel) and cerebellum (right panel) staining patterns. **(B)** Upper panels show patient 1's CSF (P1) reactivity on commercial IIFA and in-house IIFA GABA$_B$R transfected cells; lower panels show patient 2's CSF (P2) reactivity on commercial IIFA (negative staining) and in-house IIFA (positive staining) GABAbR transfected cells.

(26%) with AMPAR antibodies. Overall, we obtained a similar frequency of CSF false negative results in both cohorts: 5/51 (29%) from cohort A and 16/54 (30%) from cohort B.

As LGI1 cell transfection differed between the commercial kit and our in-house IIFA (co-transfected with ADAM23), we used a modified in-house GPI-LGI1 IIFA (surface-expressing full-length LGI1 construct, without ADAM23, as reported) (18). We tested again the 11 discordant commercial/in-house IIFA CSF samples of Cohort A (7), and Cohort B (4), and all were found negative by GPI-LGI1 IIFA, suggesting that LGI1 antibody detection in the CSF requires ADAM23 co-expression (**Figure 3**). Samples were also negative on cells transfected only with ADAM23 (data not shown). We found no alternative explanation for the lower detection of GABA$_B$R and AMPAR antibodies with the commercial IIFA.

Next, we reviewed the demographic and baseline clinical features of patients with GABA$_B$R/LGI1/AMPAR antibodies in the CSF of Cohort A (51 samples of 48 patients; 40 with available information) and Cohort B (54 patients). **Table 1** shows the comparison between patients with concordant (66) and discordant (28) commercial/in-house IIFA results for each antibody. We found that patients with discordant GABA$_B$R antibody detection (positive by in-house and negative by commercial IIFA) developed less frequently the full-blown clinical encephalitis syndrome than those with a concordant detection (positive by both IIFAs). In the discordant group 3 patients had refractory epileptic seizures and 1 had chorea (4/11) when their samples were sent for study, whereas in the concordant group 1/21 had refractory seizures (p=0.011). Additionally, brain MRI from patients with discordant GABA$_B$R and AMPAR IIFA results were more often normal at the time antibodies were determined (**Table 1**). Other clinical features (age, gender or tumor association) were not different between patients with concordant and discordant antibody results.

DISCUSSION

Our study shows that neuronal surface antibody detection using only the commercial IIFA has limitations in the diagnosis of autoimmune encephalitis. Among 241 samples that were positive by brain immunohistochemistry but negative by the commercial kit, we found 42 (18%) that had well-defined neuronal surface antibodies using our in-house IIFA. Half of these samples had antibodies against antigens not included in the commercial kit. However, the remaining 50% of samples were considered false

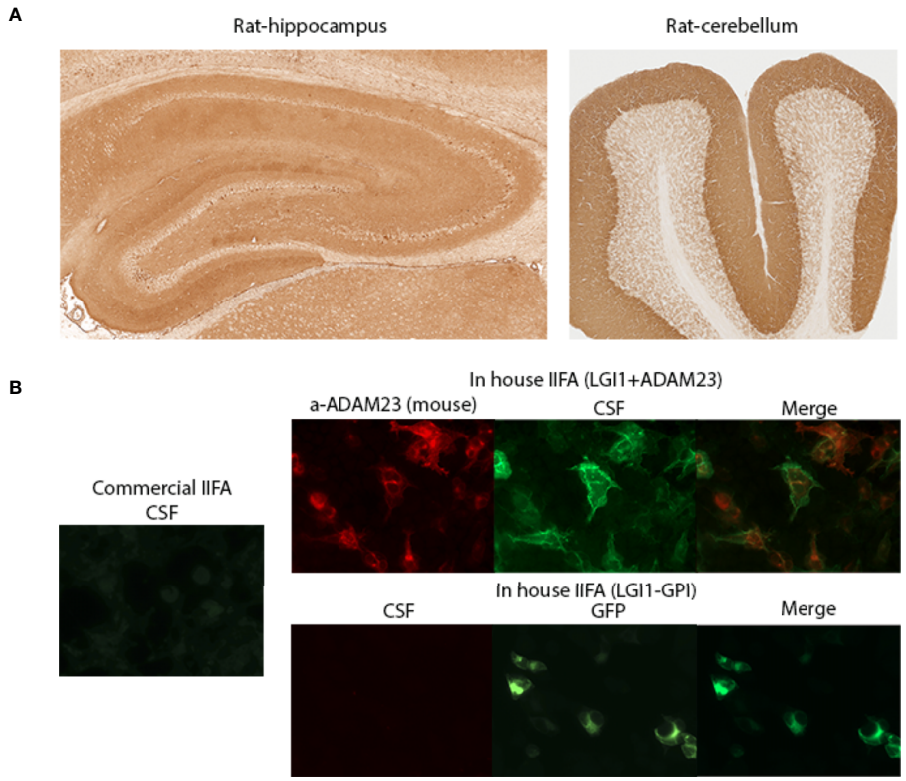

FIGURE 3 | Discrepancies identifying LGI1 antibodies by IIFA. **(A)** A patient's CSF demonstrating LGI1 immunoreactivity on rat brain immunohistochemistry. Hippocampus (left panel) and cerebellum (right panel) staining patterns. **(B)** Left panel shows patient's CSF reactivity on commercial IIFA LGI1 transfected cells (negative staining); Right panels show CSF reactivity on in-house IIFAs using LGI1 plus ADAM23 transfected cells (upper panels; positive staining) and GPI-LGI1 transfected cells (lower panels, negative staining). GPI: glycosyl-phosphatidylinositol anchored LGI1 (to display the protein on the cell surface).

TABLE 1 | Clinical features of patients with antibodies in the cerebrospinal fluid, and comparison between those with concordant and discordant results.

	Concordant in-house/commercial IIFA	Discordant in-house/commercial IIFA	p value
LGI1+	27 (19 [A] + 8 [B])	10 (6 [A] + 4 [B])	
Median age	65 y (range: 43-88)	59 y (range: 36-83)	0.352
Male sex	15 (55%)	5 (50%)	1.00
Encephalitis	23 (85%) *3 seizures, 1 Morvan's syndrome	10 (100%)	0.557
Abnormal MRI	11/16 (69%)	4/6 (67%)	1.00
Tumor	2 (7%) **1 breast cancer, 1 thymoma	1 (10%) **1 colon adenocarcinoma	1.00
$GABA_BR$+	21 (5 [A] + 16 [B])	11 (4 [A] + 7 [B])	
Median age	63.5 y (range: 24-78)	57 y (range: 36-81)	0.942
Male sex	12 (57%)	5 (45%)	0.712
Encephalitis	20 (95%) *1 refractory seizures	6 (55%) *4 refractory seizures, 1 chorea	**0.011**
Abnormal MRI	11/16 (69%)	2/9 (22%)	**0.041**
Tumor	9 (43%) **5 lung cancer, 2 neuroendocrine of unknown origin, 1 gastric, 1 vesical	4 (36%) ** 3 lung, 1 breast cancer	1.00
AMPAR+	18 (4 [A] + 14 [B])	7 (2[A] + 5[B])	
Median age	58 y (range: 21-83)	54 y (range: 27-81)	0.981
Male sex	9 (50%)	3 (43%)	1.00
Encephalitis	17 (94%) *1 refractory seizures	6 (86%) *1 refractory seizures	0.490
Abnormal MRI	10/12 (83%)	1/5 (20%)	**0.028**
Tumor	11 (61%) **5 lung cancer, 4 thymoma, 1 breast, 1 teratoma	4 (57%) **3 lung cancer, 1 teratoma	1.00

A, patients from Cohort A; B, patients from Cohort B; IIFA, indirect immunofluorescence assay; MRI, magnetic resonance image; y, years.
**Other clinical presentations; **Tumor types.*
In bold: Statistical significance $p < 0.05$.

negative results, as the in-house IIFA detected antibodies that should had been detected by the commercial IIFA. False negative results were more common in CSF (29% for CSF vs. 10% for serum), and particularly occurred for the antibodies against $GABA_BR$ (39% of cases), LGI1 (17%) and AMPAR (11%). Similar results were obtained in a separate series of 54 CSF

samples (Cohort B) from patients with LGI1, $GABA_BR$ or AMPAR antibodies (detected by brain immunohistochemistry and in-house IIFA) showing that the commercial kit failed to detect 33% of LGI1, 30% of $GABA_BR$, and 26% of AMPAR antibodies.

These results agree with previous reports based on commercial IIFA, describing a higher sensitivity for LGI1 and $GABA_BR$ antibody detection in the serum of patients with autoimmune encephalitis. For example, a study assessing 256 patients with LGI1/CASPR2 antibodies with the same commercial kit found lower sensitivity in CSF than in serum testing. Among 196 patients with LGI antibodies the authors found commercial IIFA positivity in 63% of CSF samples (24 of 38), and recommended that serum should be tested for determination of LGI1 antibodies by IIFA (19). In a second series of 38 patients with anti-LGI1 encephalitis, CSF was available for testing in 17 patients and only 9 (53%) were positive whereas all sera tested positive on the commercial IIFA (20).

A probable reason for false negative results in LGI1 antibody commercial testing is that the commercial kit does not use ADAM23 co-transfection (a presynaptic protein that forms a complex with LGI1 and interacts with voltage-gated potassium channels Kv1.1). When we re-tested the 11 CSF samples with discordant commercial/in-house LGI1 antibody results using GPI-LGI1 IIFA (cells transfected only with LGI1) all of them were found negative. An explanation could be that the recognition of some LGI1 target epitopes by CSF antibodies is improved when ADAM23 (a protein that normally interacts with LGI1) is co-expressed. The reasons for the disparity in the results of $GABA_BR$ and AMPAR antibody testing between commercial and in-house IIFAs are unclear. In a study reporting 32 patients with $GABA_BR$ encephalitis, antibody detection by commercial IIFA was less sensitive in CSF (16/20 positive) than in serum (29/30 positive). CSF results were slightly improved using in-house IIFA (18/20 positive) and with a modified assay co-transfecting the intracellular accessory protein of the B2 subunit of the GABA receptor potassium channel tetramerization domain-containing 16 (KCTD16) (20/20 positive) (21). Additionally, KCTD16 antibodies were identified in 72% of patients with anti-$GABA_BR$ encephalitis, using in-house IIFA on cells transfected only with KCTD16, and their presence indicated a higher association with lung cancer.

Finally, we compared the general clinical features between patients with concordant and discordant CSF commercial/in-house IIFA results focusing on the three antibodies that were more frequently misdiagnosed. Overall, patients with LGI, $GABA_BR$ or AMPAR antibodies had clinical features of the encephalitis typically associated with the corresponding antibody (median age 56-62 years, slight male predominance in anti-LGI1 and anti-$GABA_BR$, and higher tumor association in anti-$GABA_BR$ and anti-AMPAR, 40-60%) regardless of the negative findings with the commercial kit (7, 10, 11). Patients with false $GABA_BR$ antibody results on the commercial kit had a higher frequency of refractory seizures as main clinical presentation, without prior cognitive or behavioral changes, but were not different in demographic characteristics or frequency of paraneoplastic cases.

A limitation of our study is that we did not evaluate the specificity of the commercial IIFA. Rat brain immunohistochemistry was used as a first step for neuronal surface antibody screening and all samples tested by commercial or in-house IIFAs had positive reactivity on tissue. In the case of NMDAR antibodies, detection by immunohistochemistry is known to be more sensitive than IIFA and previous studies found that the combination of rat brain immunohistochemistry and IIFA improved diagnostic accuracy in the evaluation of neuronal surface antibodies (5, 22). Several studies using commercial kits have reported NMDAR antibodies in serum of patients with many diseases different from anti-NMDAR encephalitis as well as in healthy persons (23–25). We did not systematically test all samples received by commercial IIFA so we do not know the frequency of false positive NMDAR antibody results that occur when the commercial kit findings are not confirmed by other techniques (rat brain immunohistochemistry or in house IIFA). Another limitation is the low frequency of some antibodies, such as DPPX, for which the diagnostic value of the commercial kit could not be assessed.

The main message of our study is that the commercial IIFA for autoimmune encephalitis leads to false negative results in a substantial number of patients, especially when CSF is used, and predominantly for LGI1, $GABA_BR$ and AMPAR antibodies. Overall, the implications are important given that 1) anti-LGI1 encephalitis is the most common encephalitis in adults, but up to 13% develop cognitive impairment without criteria of encephalitis (26). A false negative result in these patients may lead to erroneously rule out the diagnosis; 2) for antibodies such as $GABA_BR$ and AMPAR the lack of detection may represent missing an underlying tumor (lung cancer, breast cancer or thymoma); 3) delaying or not giving appropriate immunotherapy may impact patients' outcome; and 4) studies on incidence, prevalence and biology of encephalitis, and recommendations about diagnosis are frequently based on results using commercial kits, and not on the real data of the disease when antibodies are comprehensively tested and results validated. Future studies investigating autoimmune encephalitis should consider these limitations. In case of negative results with the commercial kit, we recommend to extend the study using brain immunohistochemistry and in-house IIFA.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved by the ethics committee of Hospital Clínic of Barcelona. Patients' samples were coded and clinical information was anonymized prior to analysis. Written inform consent was not required as the study was observational, and the detection of neuronal surface antibodies was requested as part of

the routine diagnostic work-up. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

EM-H designed the study and wrote the manuscript in collaboration with RR-G, AS, JD, and FG. RR-G, GM-S, LN, LS, MG, and EM-H performed laboratory work and/or data analysis. All authors contributed to the article and approved the submitted version.

FUNDING

Fondo Europeo de Desarrollo Regional (FEDER) – Instituto de Salud Carlos III (Juan Rodés grant JR17/00012, EM-H; FIS PI20/00197, JD and Proyecto Integrado de Excelencia 16/00014, JD); Resident award "Josep Font" by Hospital Clínic Research, Innovation and Education Departments (MG); La Caixa Foundation (JD); Edmond J Safra Foundation (JD); Fundació CELLEX (JD) and Red Española de Esclerosis Múltiple RD16/0015/0002, RD16/0015/0003 (AS).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.691536/full#supplementary-material

Supplementary Table 1 | Antibody detection by in-house and commercial assays in patients with paired serum and cerebrospinal fluid from Cohort A.

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Conflict of Interest: JD receives royalties from Athena Diagnostics for the use of Ma2 as an autoantibody test and from Euroimmun for the use of NMDA, GABAB receptor, GABAA receptor, DPPX and IgLON5 as autoantibody tests. FG holds a patent for the use of IgLON5 as an autoantibody test.

The remaining 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.

The handling editor declared a past co-authorship with the authors FG and JD.

frontiers in Neurology

ORIGINAL RESEARCH
published: 30 June 2021
doi: 10.3389/fneur.2021.690415

Antibody Testing for Neurological Autoimmune Disorders: Evaluation of Best Practices at a Tertiary Referral Center

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Background: Autoimmune neurology is a rapidly evolving field of study, where best practices for neurological antibody testing have yet to be determined. The growing number of options for antibody panel testing can create confusion amongst ordering clinicians and lead to ordering several concurrent panels (i.e., overlapping evaluations) or repeat panel evaluations. This study determined the frequency of these evaluations for autoimmune and paraneoplastic disorders and investigated how these practices informed clinical decision making and management.

Methods: This was a retrospective observational study of adult patients presenting to University of Texas Southwestern (UTSW) in 2017 with requests for antibody panels for autoimmune encephalitis and paraneoplastic disorders. Individuals with more than one panel requested were defined as either an overlapping evaluation (more than one panel requested within 14 days) or repeat evaluation (more than one panel requested 14 or more days apart). For those individuals with repeat panel testing, the proportion of panels with a change in antibody status or subsequent changes in clinical diagnosis and decision making were recorded.

Results: There was a total of 813 panels sent on 626 individuals. Twenty percent (126 individuals) had more than one panel requested. Only 10% of individuals had a matched serum and CSF evaluation. Forty-seven overlapping evaluations were performed in 46 (7.3%) of the individuals studied. Fifty-four (8.6%) individuals underwent 70 repeat evaluations encompassing 79 panels (9.7% of total panels ordered). Ten repeat evaluations showed a change in antibody status, of which only two were clinically significant. There was a single case where clinical management was affected by repeat autoantibody evaluation.

Conclusions: Ordering practices for suspected autoimmune encephalitis and paraneoplastic disorders are suboptimal with frequent overlapping antibody panel evaluations and non-paired serum/CSF samples at our center. Repeat autoantibody testing is a commonplace practice yet yielded novel information in only a minority

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Edited by:
John Greenlee,
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Reviewed by:
Sudarshini Ramanathan,
The University of Sydney, Australia
Ricardo Constantino Ginestal,
Hospital Clínico San Carlos, Spain

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

Received: *02 April 2021*
Accepted: *04 June 2021*
Published: *30 June 2021*

Citation:
Fredrich SE, Vernino S and Blackburn KM (2021) Antibody Testing for Neurological Autoimmune Disorders: Evaluation of Best Practices at a Tertiary Referral Center. Front. Neurol. 12:690415. doi: 10.3389/fneur.2021.690415

of cases. These new results were, as a rule, clinically irrelevant and changed clinical decision making in <1% of cases. There is limited utility in these practice patterns. Future efforts should be directed at the development and standardization of neurological autoimmune and paraneoplastic autoantibody testing practice standards.

Keywords: autoimmune neurology, stewardship, antibody panels, evaluation of paraneoplastic disorders, evaluation of encephalitis, repeat testing, utilization, practice patterns

INTRODUCTION

Autoimmune neurology is a rapidly evolving field of study, largely fueled by the discovery of autoantibodies targeting neuronal and glial proteins. Antibody-mediated neurological disorders typically present with severe, progressive neurological symptoms, and timely treatment with immunotherapy can result in dramatic improvement and favorable long-term outcomes (1). Antibody testing for autoimmune and paraneoplastic neurological disorders is available through several commercial laboratories in the United States. Best practices for neurological antibody testing have not been defined, but it is generally advised that both serum and cerebrospinal fluid (CSF) samples be submitted for testing, as certain antibodies are more sensitive in CSF (2). In addition, different neuronal autoantibodies can present with overlapping clinical features, antibody "panels" are commonly ordered for suspected autoimmune neurological disorders. The growing options for antibody testing can create confusion amongst ordering clinicians, leading them to order multiple panels during a single encounter to ensure a comprehensive evaluation. Additionally, antibody testing is occasionally repeated in the same patient during a future patient encounter. Whether these practices increase detection of antibody-mediated neurological disorders has not been systematically examined. In this study, we determined the frequency of overlapping and repeat antibody testing for autoimmune and paraneoplastic disorders in patients presenting to the University of Texas Southwestern Medical Center (UTSW) and investigated how these practices informed clinical decision making and management.

MATERIALS AND METHODS

The study was approved by the UTSW institutional review board. This was a retrospective observational study of adult patients (>18 years old) presenting to UTSW in 2017 with requests for autoimmune encephalitis, epilepsy, dementia, and paraneoplastic disorders antibody panels sent to Mayo Medical Laboratories[1]. These patients were cross matched with a list of requests for antibody testing submitted to the UTSW clinical laboratory from 2011 to 2020. Patients from 2017 with more than one antibody test requested during this period were included in the final analysis (see **Figure 1**).

Instances where more than one panel was requested were placed in one or more of the following categories: paired serum and CSF evaluation, overlapping evaluation, and/or repeat evaluation. A paired serum and CSF evaluation was defined as a request for serum and CSF panels obtained within 14 days of each other. An overlapping evaluation was defined as multiple requests for antibody panels obtained <14 days apart, excluding paired serum and CSF evaluations. A repeat evaluation was defined as more than one antibody panel obtained ≥14 days apart. Accidental reordering was defined as multiple requests for identical antibody panels <14 days apart. The 14-day time period was chosen to demarcate overlapping and repeat testing as, in our experience, results from initial testing tend to return in this timeline.

For those individuals with repeat autoantibody evaluations, the results of the panel testing were recorded. The number of results with a change in antibody status (e.g., going from antibody positive to negative) was tabulated. A single evaluation was considered positive if any of the individual panels returned with a positive result. If a single individual had more than one repeat evaluation, the additional repeat evaluations were compared to the most recent evaluation for the purpose of determining change in antibody status. If an antibody status change was found, this was sorted into one of four categories based on comparison of identical or non-identical panels and positive to negative or negative to positive panel transition (see **Figure 1**). The time between repeat evaluations was determined by the date of the last panel in the first evaluation to the date of the first panel in the second evaluation.

For individuals who had one or more repeat evaluations with a change in autoantibody status, medical records were reviewed for changes in clinical diagnosis and medical decision making based on repeat panel results. Changes in medical decision making were defined as one or more of the following: starting, stopping, or changing immunotherapies, starting or stopping anti-seizure medications (ASM) in a patient who presented with seizure, ordering a malignancy screening consisting of positron emission tomography (PET) scan and/or computed tomography (CT) scan of the chest, abdomen, and pelvis, within 30 days of panel results. Clinical diagnoses were abstracted from neurology consultation notes and billing codes.

RESULTS

There was a total of 768 antibody panels submitted on 626 individuals in 2017. The average age of this group was 57.2 years (ranging from 18 to 93 years old) with slight female predominance (53% of individual). Ultimately 24 cases were due to autoimmune or paraneoplastic disorders. One

[1]Panels requested include PAVAL, ENS1, ENC1, EPC1, EPS1, PAC1, ENCEC, EPIES, EPIEC, ENCES, DEMEC, ENS2, EPS2, DEMES. For a full description of panels, visit www.mayocliniclabs.com/test-catalog.

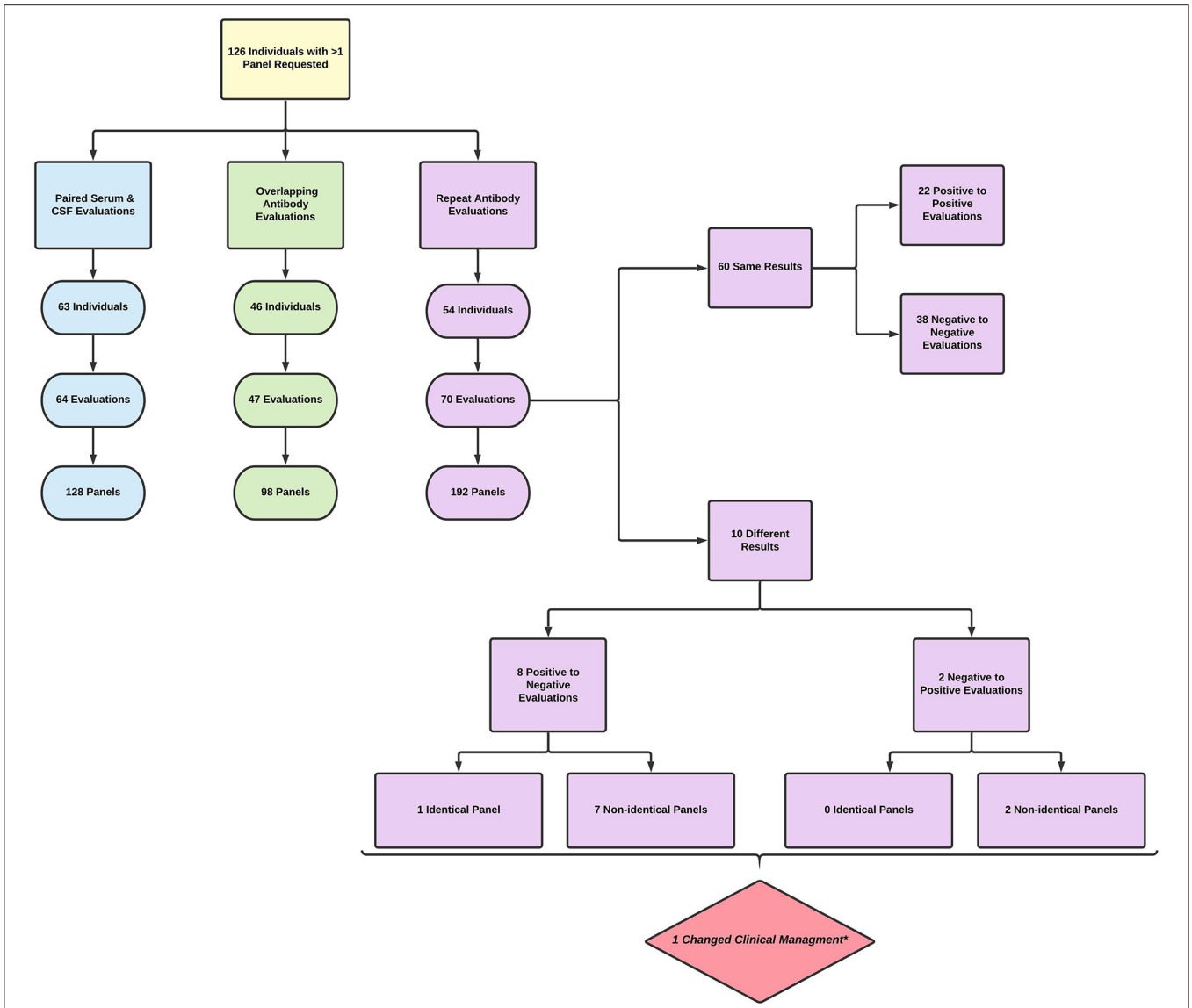

FIGURE 1 | Flowchart of results for individuals with >1 antibody panel requested.

hundred and twenty six individuals (20.1%) had more than one panel requested (see **Figure 1**). The proportion of panels and evaluation types can be seen in **Figures 2**, **3**. There were an additional 45 panels ordered as repeat antibody evaluations from 2011 to 2020 on these individuals. In total, 290 panels (35.7% of all panels studied) were requested as part of an overlapping or repeat panel evaluations (see **Table 1**). Six patients had both overlapping and repeat evaluations performed.

Paired Serum and CSF Evaluations

There were 64 paired serum and CSF evaluations (128 panels, 15.7%). Only 10.1% of individuals had a paired serum and CSF evaluation. One individual had two paired serum and CSF evaluations performed.

Overlapping Evaluations

Forty-seven overlapping evaluations were performed in 46 (7.3%) of the individuals studied. Overlapping evaluations included 98 panels (12.1% of total tests requested). Of these, 17 (36%) overlapping evaluations were requested on identical panels. While the majority of overlapping evaluations consisted of two panels per evaluation, four individuals had overlapping evaluations with three panels ordered. One individual additionally had two overlapping evaluations.

Repeat Evaluations

Fifty-four (8.6%) individuals underwent 70 repeat evaluations encompassing 79 repeat panels (9.7% of total panels ordered). The average time between repeat evaluations was 350 days (median 200 days, range 140–1,941 days). Identical panels were

requested as a part of 41 (58.6%) repeat evaluations.Eight individuals underwent more than one repeat evaluation, with two individuals having three repeat evaluations. Eighty-six percent of repeat evaluations did not show a change in autoantibody status, with 97% of initial negative evaluations remaining negative.

Ten repeat evaluations had a change in antibody status (**Figure 1**). Eight repeat evaluations (11.4%) changed from positive to negative. One evaluation compared identical panels and seven compared non-identical panels. Two evaluations (2.8%) changed from negative to positive; in both instances, a different panel was ordered in the repeat evaluation (**Table 2**).

The transition in antibody status changed clinical management in two cases. In the first case, ASMs were stopped in a patient with leucine-rich glioma-inactivated 1 (LGI1) antibody associated seizures following panel transition from positive to negative. For the second case immunotherapy was started in an individual presenting with encephalopathy following a new positive glutamic acid decarboxylase 65 (GAD-65) antibody. There were no cases in which new antibody panel results correlated to a change in clinical diagnosis or prompted a malignancy screening.

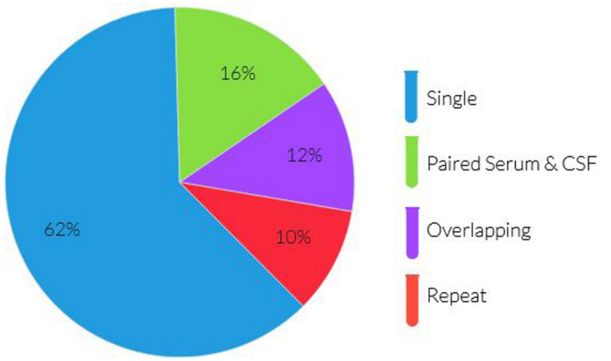

FIGURE 2 | Proportion of panels by evaluation type.

DISCUSSION

Overlapping and repeat evaluations for suspected autoimmune and paraneoplastic disorders occurred frequently at our medical center, occurring in 15.9% of patients with testing in 2017. The results of these evaluations rarely differed from the initial evaluation and seldom influenced clinical management. Our study suggests that, at our center, there is wide variability in practice habits surrounding autoantibody evaluations, reflecting the increasingly complex nature of autoantibody testing.

TABLE 1 | Number of individuals, evaluations, and panels performed by evaluation type.

Evaluation type	Number of individuals (%)	Number of evaluations (%)	Number of panels (%)
Single panel	500 (79.9%)	500 (67.8%)	500 (61.5%)
Paired serum & CSF	63 (10.1%)	64 (8.7%)	128 (15.7%)
Overlapping	46 (7.3%)	47 (6.4%)	98 (12.1%)
Repeat	54 (8.6%)	70 (9.5%)	79 (9.7%)
Identical panels	48 (7.7%)	57 (7.7%)	58 (7.1%)

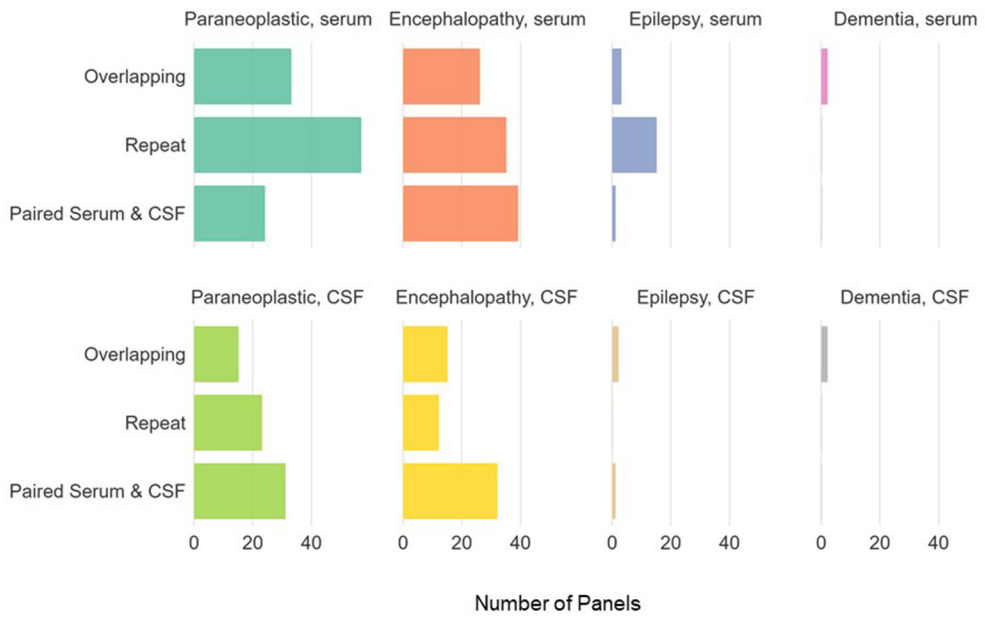

FIGURE 3 | Panel types ordered for individuals with >1 antibody panel requested. Paraneoplastic panels include test codes PAVAL, PAC1. Encephalopathy panels include test codes ENS1, ENS2, ENC1, ENCEC, ENCES. Epilepsy panels include test codes EPC1, EPS1, EPS2, EPIES, EPIEC. Dementia panels include test codes DEMEC, DEMES.

TABLE 2 | Repeat evaluations with a change in antibody status.

	Patient	Clinical presentation	Panel 1 (result, level)	Panel 2 (result, level)	Time between evaluations (months)	Management changes
Positive to negative, non-identical panels	Patient 1	Seizures and Encephalopathy	Encephalopathy Panel, serum (GAD-65: 0.05 nmol/L)	Epilepsy Panel, serum (negative)	5	None
	Patient 2	Seizures	Encephalopathy Panel, serum (VGKC: 0.22 nmol/L, LGi1: positive)	Epilepsy Panel, serum (negative)	11	Patient tapered off ASM following repeat negative evaluation
	Patient 3	Myopathy with anti-Ku antibodies	Encephalopathy Panel, serum (GAD-65: 0.19 nmol/L)	Paraneoplastic Panel, serum (negative)	3	None
	Patient 4	Diarrhea, weight loss, and behavior changes	Paraneoplastic Panel, serum (ARBi: 5.41 nmol/L)*	Paraneoplastic Panel, CSF (negative)	0.5	None
	Patient 5	Memory impairments	Encephalopathy Panel, serum (GAD-65: 0.08 nmol/L)	Encephalopathy Panel, CSF (negative)	3	None
	Patient 6	Encephalopathy with memory impairments	Epilepsy Panel, serum (ARBi: 0.05 nmol/L)	Encephalopathy Panel, serum and Paraneoplastic Panel, CSF (negative)	0.5	None
	Patient 7	Encephalopathy in setting of phosphaturic mesenchymal tumor	Paraneoplastic Panel, CSF (gAChR: 0.3 nmol/L)	Encephalopathy Panel, serum (negative)	0.5	None
Positive to negative, identical panels	Patient 8	Encephalitis in setting of parotid carcinoma	Encephalopathy Panel, serum (GAD-65: 0.08 nmol/L)	Encephalopathy Panel, serum (negative)	11	None
Negative to positive, non-identical panels	Patient 9	Autonomic dysfunction	Paraneoplastic Panel, serum (negative)	Encephalopathy Panel, serum (GAD-65: 0.20 nmol/L)	16	None
	Patient 10	Encephalopathy with psychiatric symptoms	Encephalopathy Panel, CSF (negative)	Encephalopathy Panel, serum (GAD-65: 0.07 nmol/L)	6	Plasmapheresis initiated following GAD-65 antibody level**

ARBi, Acetylcholine receptor binding antibody; gAChR, Alpha 3-ganglionic acetylcholine receptor antibody; GAD-65, Glutamic acid decarboxylase 65 antibody; LGI1, Leucine-rich glioma-inactivated 1 antibody; VGKC, Voltage gated potassium channel antibody.

**Antibodies to dipeptidyl-peptidase-like protein-6 (DPPX) identified in this patient, but not reported on commercial test result.*

***Immunotherapy change based off change in clinical symptoms.*

Generally agreed upon practice standards include concurrent serum and CSF evaluations for suspected central nervous system autoimmune disorders—a practice that was only implemented a fraction of the time in this study. Even among the paired serum and CSF evaluations, panels were ordered in a "mix and match" fashion (e.g., epilepsy panels in the serum with encephalitis panels in the CSF). The vast majority of unmatched panels (84.3% of panels evaluated) were serum panels. There are numerous potential explanations for serum testing lacking a matched CSF sample. Lumbar punctures are invasive, time consuming procedures, which some patients may refuse in the outpatient setting. Additional medical factors, including body habitus, infection risk, and mental status, may limit the ability to perform a lumbar puncture. Lastly, each antibody panel requires a minimum volume of CSF for evaluation; often infectious and neoplastic studies are additionally needed from the same CSF sample. If the amount of CSF required for the intended studies is not calculated prior to the lumbar puncture, clinicians may have to choose between studies or risk a repeat procedure.

The practice of ordering overlapping evaluations—herein defined as multiple requests for antibody panels obtained <14 days apart, excluding paired serum and CSF evaluations—may reflect several factors. First, ordering clinicians are presented with a myriad of options when considering panel evaluations. This is compounded by frequent updates to panel contents and the interval development of panels for new indications. Anecdotal experience with clinicians suggests significant confusion regarding which panel is appropriate in a given scenario. All of these factors may lead clinicians to order multiple antibody panels in order to adequately evaluate patients with severe neurological impairment. Indeed, studies reviewing physician ordering practices and social consequences show a relative increase in false positive errors compared to false negative- implying clinicians regret the consequences of omitting

testing that may lead to benefit more than the consequences of unnecessary testing (3). Comprehensive evaluation does not necessitate ordering multiple antibody panels, however; several established laboratories utilize immunohistochemistry as an initial screening tool which will detect known and unknown antibody binding. Patient 4 from **Table 2** provides an example of this; at initial evaluation the patient was found to have an unidentified binding pattern on immunohistochemical staining. This was confirmed years later to be dipeptidyl-peptidase-like protein-6 (DPPX) antibodies, which was not commercially available at time of initial panel testing. Accidental reordering is an additional factor, as evidenced by 36% of overlapping evaluations consisting of identical panels in this study. Developing clinical decision support tools in electronic health records may be a viable strategy to mitigate many sources of overlapping evaluations.

The results of repeat evaluations rarely differed from those of initial testing. In this study, clinically insignificant GAD-65 antibodies (determined by low antibody levels and incompatible clinical phenotypes) represented the majority of antibody status conversions. GAD-65 antibodies are known to be present at low levels in healthy controls, systemic inflammatory or autoimmune disorders, and unrelated neurologic diseases (4, 5). When negating clinically insignificant GAD-65 antibodies, four repeat evaluations transitioned from positive to negative and zero transitioned from negative to positive. The latter indicates that initial negative autoantibody evaluations are sufficient for autoimmune and paraneoplastic antibody surveillance. Repeating identical testing follow initial negative evaluation is largely a flawed practice; management decisions should be influenced more heavily by clinical presentation with ancillary testing results than antibody status, as reflected in the 2016 consensus guidelines for diagnosing autoimmune encephalitis (6). Studies show providers appropriately initiate immunotherapy prior to panel results, however then discontinue immunotherapy following a negative antibody panel (7, 8). This practice is fraught with error as antibody negative autoimmune encephalitides are not uncommon (9).

Repeat testing following an initial positive antibody evaluation suggests a previous autoimmune or paraneoplastic disorder was known or suspected, therefore repeat testing would provide information to prompt changes to clinical management. There was a single case of seropositive conversion in which clinical management was altered, however the change in immunotherapy was based on clinical symptoms and not antibody status.

This pattern of widespread, redundant panel testing did not increase the sensitivity of detecting autoimmune or paraneoplastic diseases, nor did it alter clinical decision making. When juxtaposed against the expense of these panels, the value of this strategy in clinical practice is severely limited. Indeed, several retrospective studies have shown a significant proportion of autoantibody panels ordered are inappropriate based on clinical indication or other ancillary test results; this underscores the need for continued provider education in the field of autoimmune neurology and recognition of aggregate bias amongst ordering physicians (10). While many studies have shown low rates of antibody positivity with panel testing, increased clinical suspicion for primary autoimmune disorders improves panel sensitivity (11–13). Healthcare cost savings and autoantibody detection rates have additionally been shown to increase following the implementation of predictive likelihood scoring systems (14). Integrating decision support tools, such as the antibody prevalence in epilepsy and encephalopathy (APE2) score, into the electronic medical record and the resultant effect on physician ordering practices is an area needed in future study.

Based on the results of this study, the following recommendations for CNS autoimmune and paraneoplastic panel testing should be considered by clinicians.

1. Panel testing should be ordered as paired serum and CSF samples. This ensures the highest sensitivity for the greatest number of antibody mediated diseases—some of which are more sensitive in the CSF, others more sensitive in the serum.
2. Paired panel testing should be ordered as matching pairs. For example, encephalopathy panels in the serum will match antibodies most closely with encephalopathy panels in the CSF.
3. Overlapping panel evaluations are not advised if sending panel testing to a laboratory which performs tissue-based screening assays with reflexive testing; the screening assay will capture antibodies not specified in the panel and the appropriate reflexive testing will be performed.
4. If a clinician is considering ordering an overlapping evaluation and submitting samples to a laboratory without screening and reflexive assays, identify the antibody targets that differ between the panels. Revisit the clinical presentation of the patient and critically evaluate if the unique antibodies on either panel fit the clinical presentation of the patient.
5. Repeat autoantibody evaluations should be avoided, unless there is a significant change in the clinical status of the patient, or a specific antibody is highly suspected that was not evaluated for on the initial panel. This is especially cautioned against in individuals with prior negative autoimmune panel testing.
6. Repeat, identical antibody testing is not recommended for individuals with an appropriate initial evaluation with a negative result for the following indications: autoimmune encephalitis, epilepsy, movement disorders, and paraneoplastic disorders.
7. Repeat, identical antibody testing can be performed in seropositive individuals to assess for titer change or transition to seronegative status if there is a predetermined plan which influences clinical management, including but not limited to changes in ASMs, immunotherapies, or malignancy surveillance.
8. Isolated antibody testing should be reserved for the conditions outlined in recommendation seven; CNS autoimmune and paraneoplastic conditions should otherwise be evaluated with panel testing given the non-specific nature of presentation.

There are some limitations to our study. First, a single year of panel testing for individuals was reviewed and cross matched for repeat evaluations over a fixed time frame at our institution. It is possible that there was additional antibody testing prior to 2011 or that individuals may have had additional testing outside

our hospital. Additionally, this retrospective observational study was performed on adult patients at a single tertiary level medical center; the results of this study and ensuing recommendations may not be generalizable to community hospitals, areas without neurologic expertise, or pediatric populations. Throughout the study time frame there were significant changes in commercially available panels, which may have altered ordering patterns. While this study did evaluate if new antibody testing results impacted clinical decision making, it did not address if repeat evaluations with identical results altered clinical decision making. Lastly, changes in clinical decision making were strictly defined for the purposes of this study and may not encompass all possible clinical decisions based on new antibody panel results.

CONCLUSIONS

Ordering practices for suspected autoimmune encephalitis and paraneoplastic disorders are suboptimal with frequent overlapping antibody panel evaluations and non-paired serum/CSF samples at our center. Repeat autoantibody testing is a commonplace practice yet yielded novel information in only a minority of cases. These new results were, as a rule, clinically irrelevant and changed clinical decision making in <1% of cases. There is limited utility in these practice patterns. Future efforts need to be directed at the development and standardization of neurological autoimmune and paraneoplastic autoantibody testing practice standards.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UT Southwestern IRB. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SF participated in the conception of the study, research design, performance of the research, data analysis, and article writing. KB participated in the research design, performance of the research, and article modification. SV participated in the research design and article modification. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: SF's fellowship is funded by the National Multiple Sclerosis Society. She has served as a consultant for EMD Serono. SV has served as a consultant for Alterity, Genentech, Catalyst and Sage Therapeutics. He has received research support from Dysautonomia International, BioHaven, Grifols and Quest Diagnostics (through a licensing contract). KB's fellowship was funded by the Siegel Rare Neuroimmune Association.

frontiers in Neurology

CASE REPORT
published: 16 July 2021
doi: 10.3389/fneur.2021.691509

A Longitudinal, Observational Analysis of Neuronal Injury Biomarkers in a Case Report of a Patient With Paraneoplastic Anti-CRMP5 Antibody-Associated Transverse Myelitis

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OPEN ACCESS

Edited by:
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Patrick Joseph Waters,
University of Oxford, United Kingdom
Harald Hegen,
Innsbruck Medical University, Austria

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

Received: 06 April 2021
Accepted: 21 June 2021
Published: 16 July 2021

Citation:
Mizenko C, Bennett JL, Owens G, Vollmer TL and Piquet AL (2021) A Longitudinal, Observational Analysis of Neuronal Injury Biomarkers in a Case Report of a Patient With Paraneoplastic Anti-CRMP5 Antibody-Associated Transverse Myelitis. Front. Neurol. 12:691509. doi: 10.3389/fneur.2021.691509

Biomarkers are needed to guide therapeutic decision making in autoimmune and paraneoplastic neurologic disorders. Here, we describe a case of paraneoplastic collapsing response-mediator protein-5 (CRMP5)-associated transverse myelitis (TM) where plasma neurofilament light (NfL) chain and glial fibrillary protein (GFAP) levels were observed over a 14-month clinical course, correlating with radiographical and clinical outcome measures in response to treatment. Blood and CSF samples obtained at diagnosis as well as 7 and 14 months into treatment. At the time of initial diagnosis, both plasma NfL (782.62 pg/ml) and GFAP (283.26 pg/ml) were significantly elevated. Initial treatment was with IV steroids and plasma exchange (PLEX) followed by neuroendocrine tumor removal, chemotherapy, and radiation. After initial improvement with chemotherapy, the patient experienced clinical worsening and transient elevation of plasma NfL (103.27 pg/ml and GFAP (211.58 pg/ml) levels. Whole body positron emission tomography PET scan did not demonstrate recurrence of malignancy. Repeat PLEX and rituximab induction resulted in improvements in patient function, neurologic exam, and plasma biomarker levels. To our knowledge, this is the first described longitudinal, prospective analysis of neuronal injury biomarkers and association of clinical treatment outcomes in CRMP5 myelitis. Our findings suggest that clinical improvement correlates with NfL and GFAP concentrations.

Keywords: paraneoplastic, CRMP5 antibody, biomarker, neurofilament light, myelitis

INTRODUCTION

Neuronal Injury Biomarkers

Identification and quantification of neuroaxonal damage may improve diagnostic accuracy and treatment of autoimmune and paraneoplastic neurological disease. Biomarkers currently under investigation for monitoring neuronal injury, demonstrate promise for use in multiple clinical settings (1). The cytoskeleton of central nervous system (CNS) neurons is composed of

intermediate neurofilaments (Nfs) composed of four heterologous polypeptidens: alpha-internexin, neurofilament light chain (NfL), neurofilament medium chain (NfM), and neurofilament heavy chain (NfH). Following neuronal injury, the concentrations of these neurofilament proteins are elevated in cerebral spinal fluid (CSF) and blood. Most, if not all neurodegenerative diseases will result in an elevation in blood Nf levels, providing a general indicator of axonal damage. Since NfL is more highly expressed than other Nf polypeptides, Nfl has now become the preferential candidate biomarker for following axonal injury (1).

Other neuronal biomarkers may provide alternative information on CNS injury in neuro-immunological disorders. A highly localized cytosolic protein in neurons and neuroendocrine cells, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), is involved in neuronal repair after injury through removal of abnormal proteins *via* the ubiquitin-proteasome pathway, autophagy, and regulation of synaptic function. Indeed, prior research has demonstrated a correlation between levels of UCH-L1 and intracranial injury (2).

Glial fibrillary acidic protein (GFAP) is a brain-specific intermediate filament expressed in astrocytes and ependymal cells. Increased levels of GFAP may reflect astrogliosis, upregulated expression, glial scarring, and astrocyte destruction (3–5). Serum levels of GFAP and NL are likely to be good biomarkers for disease activity and disability in neuromyelitis optic spectrum disorders (NMOSD) (6). Furthermore, in patients enrolled in N-MOmentum trial (NCT2200770), serum GFAP levels increased significantly within 1 week of an NMOSD attack in placebo-treated patients (5). In multiple sclerosis (MS), studies propose GFAP as a biomarker of disease progression (7, 8) based on observation as a correlate between GFAP levels in CSF and neurologic disability assessed by Expanded Disability Status Score (EDSS) and Multiple Sclerosis Severity Scale (MSSS) (9). Additional reports of serum GFAP levels as brain-specific marker for malignant gliomas (10).

Tau protein is a heat stable, microtubule-associated protein (MAP) essential for inducing microtubules from tubulin and stabilization of cytoskeleton scaffolding; the absence of tau *in vitro* prevents tubulin from assembling into microtubules (11, 12). One main function of tau is the stabilization and flexibility of axonal microtubules; abnormal tau deposition is common in neurodegenerative diseases (12). In Alzheimer's disease (AD) and dementia, both tau and beta-amyloid pathology, have been correlated with blood NfL levels (13). A large prospective study showed that plasma tau levels associate with worsening cognition, atrophy, and hypometabolism during follow-ups despite no clear association between tau and NfL (13). However, increases of plasma NfL concentrations in combination with reduced plasma beta-amyloid strongly associated with higher risk of developing dementia and AD dementia (13).

Paraneoplastic Neurological Disorders

Paraneoplastic neurological disorders (PNDs) are immune-mediated disorders that can affect any part of the neuroaxis and occur in association with cancer. This is thought to be driven by the immune response directed against proteins shared between tumor cells and neurons, thus resulting in neuronal destruction and cell death. PNDs are characterized by the detection of neuronal autoantibodies in the serum and CSF. One such autoantibody is anti-CV2/collapsing response mediator protein (CRMP)5. This protein is integral to the morphology of neurons and is highly expressed in cerebellar purkinje cells. CRMP5 neuronal antibody is often associated with lung cancer and thymoma-related autoimmunity (14). Clinical manifestations may vary and include rapidly progressive myelopathy, cerebellar ataxia, polyneuropathy, optic neuritis, and chorea (15–18). The management of CRMP5 autoimmunity focuses on diagnosing and treating the underlying tumor in combination with immunotherapy.

Since PND-like CRMP5 often results in progressive and debilitating symptoms, early recognition is crucial to minimize irreversible neurological damage. While clinical assessments are the sole measure for assessing therapeutic response; however, novel quantitative biomarkers are needed to monitor the immune response and neuronal injury. Such biomarkers will be critical for both patient care and the design of robust randomized controlled trials.

In this longitudinal evaluation of a single female patient with anti-CRMP5-associated transverse myelitis (TM), we assessed four plasma markers of neuronal injury including NfL, GFAP, tau, and UCH-L1 in relation to neurologic symptoms and radiographic disease activity during her clinical and treatment course.

METHODS

The patient was enrolled in the Autoimmune, Paraneoplastic, and Inflammatory Neurological Disease (APIND) Patient Registry at the University of Colorado. The study was approved by the Institution Review Board of the University of Colorado, Aurora, CO. As part of the APIND, clinical data and biorepository specimens were collected prospectively over time at each follow-up visit. Biorepository specimens collected included CSF at the time of diagnosis as well as longitudinal serum samples. Samples are stored at −80°C.

Patient specimens were collected at the time of clinical presentation (time point 0), as well as 6 and 14 months into her clinical course. Sample collection at time point 0 occurred prior to the administration of any therapeutic agents including the initiation of plasma exchange.

Serum and CSF levels of tau, GFAP, UCH-L1, and Nfl were measured using the Quanterix Small Immunoassay (Simoa) Neurology 4-PlexA assay. Normal values were provided by Quanterix for plasma, serum, and CSF (**Supplementary Table 1**) (19). The assay performed for internal control and quality assurance of Quanterix machine and N4PA. Using Person sample correlation coefficient, self-validation yielded high correlation between the two plasma samples of 0.998651. Adjusting for the small sample size R-squared was 0.997303.

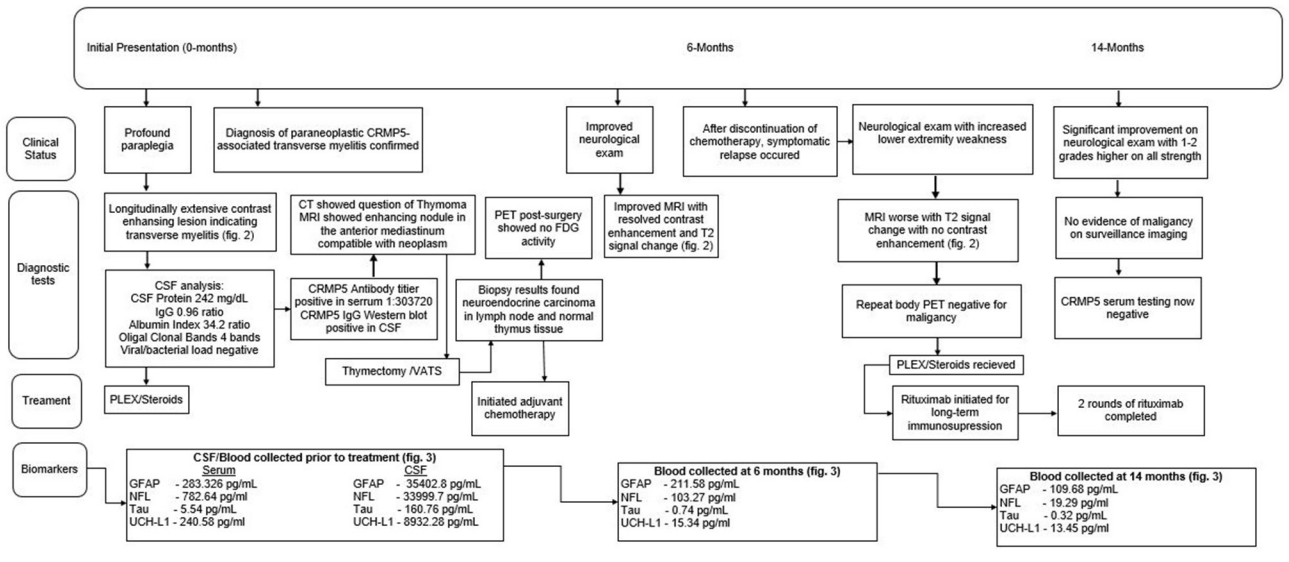


FIGURE 1 | Timeline of clinical course, diagnostic testing, treatment, and biomarker sampling.

RESULTS

Clinical Case Report

A 54-year-old female with a 39-pack-year history of tobacco use and a history of rheumatoid arthritis presented to an outside hospital with a history of progressive lower extremity weakness (**Figure 1** for timeline of events). Her recent history was notable for worsening back pain over the prior 5 months. She denied any sensory loss but endorsed urinary frequency and hesitation. She had an abrupt worsening of her motor weakness occurring over days leading to transfer to our institution.

Initial neurological exam was remarkable for profound lower extremity weakness (0/5) throughout, with the exception of 1/5 dorsiflexion in the right ankle and bilateral toes. In the upper extremities, she had full strength on the right and mild extensor weakness on the left (4/5). She had diffuse hyperreflexia, more profound and on the right, with bilateral clonus and Hoffmann's sign.

Magnetic resonance imaging (MRI) showed a contrast-enhancing, longitudinally extensive lesion from C3 down to T12 (**Figure 2**). Brain MRI was unremarkable.

CSF analysis demonstrated a lymphocytic predominant pleocytosis of 93 nucleated cells/μl [reference range (ref): <5 cells/μl], elevated protein of 242 mg/dl (ref: <45 mg/dl), elevated immunoglobulin-G (IgG) index of 0.96 (normal ratio: 0.28–0.66), and four CSF-restricted oligoclonal bands (OCBs). Infectious work up on the CSF was negative for herpes simplex virus (HSV), varicella zoster virus (VSV), arbovirus panel (including West Nile virus), and enterovirus.

The patient's history, neurological exam, and radiographic findings consistent with transverse myelitis, with a high degree of suspicion for autoimmune or paraneoplastic etiology including neuromyelitis optic spectrum disorder (NMOSD) or other inflammatory etiology such as neurosarcoidosis. Infectious etiologies have largely been ruled out though CSF-specific PCR and antibody testing as above. While awaiting antibody results, she was empirically treated with a 5-day course of intravenous (IV) methylprednisolone (MP) and plasma exchange (PLEX).

Her aquaporin-4 (AQP4) eventually returned negative as well as myelin oligodendrocyte glycoprotein (MOG). CRMP5 antibody returned positive in serum 1:30,720 (ref: <1:240 titer) and CRMP5 IgG Western Blot CSF Positive (Mayo Clinic Laboratories), thus, leading to a final diagnosis of paraneoplastic CRMP5-associated transverse myelitis.

Further malignancy work up with computed tomography (CT) of chest showed two inferior left thyroid lobe nodules, largest 8 mm and normal right thyroid. Soft tissue density nodule in anterior mediastinum adjacent to left brachycephalic vein and lungs indicated mild centrilobular emphysema. Biopsy results of the mediastinum nodule found the tumor to be high-grade neuroendocrine carcinoma, entirely contained in single lymph node without extranodal extensions or involvement of thymic tissue (Ki67: >90%). Follow-up positron emission tomography (PET)-CT postsurgery showed no occult flourodeoxyglucose (FDG) avidity.

Despite the inability to identify a primary tumor, given the strong association of CRMP5-IgG with neuroendocrine tumors, specifically small cell lung cancer, she started on adjuvant chemotherapy with etoposide. Two months after treatment initiation, patient's neurologic exam showed improvements with lower extremity weakness noted having antigravity movements in knee flexion and extension.

Outpatient follow-up visit occurred 5 months after presentation. She was now able to stand and transfer but still not able to walk with assistance. Motor exam continued to demonstrate improvement with the ability to move all muscle groups against gravity with proximal greater than distal weakness remaining. Repeat spinal imaging demonstrated

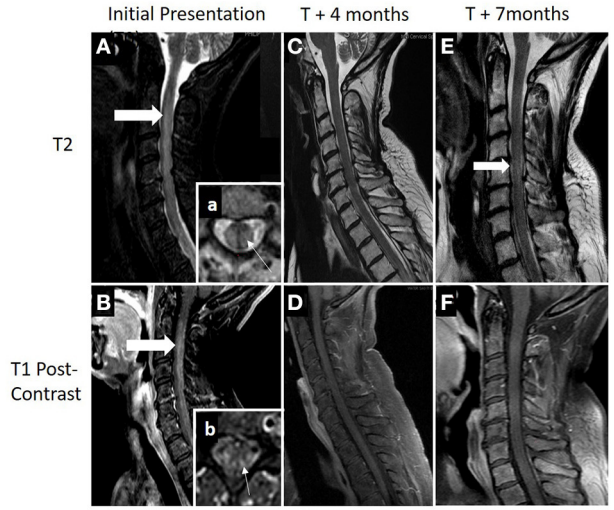


FIGURE 2 | (A,B) MRI demonstrating an longitudinally extensive T2 lesion (top row; thick white arrow) with associated contrast enhancement (bottom row; thick white arrow). Box (a), axial view showing a typical presence of transverse myelitis involving a central lesion, involving more than 2/3 of the cross-sectional area (thin white arrow). Box (b), areas of patchy contrast enhancement concentrated within areas of the posterior cord (thin white arrow). **(C,D)** Patient had clinical and radiographical improvement at 4 months after diagnosis following acute treatment with plasma exchange, high-dose steroids, and adjuvant chemotherapy. **(E,F)** Seven months after diagnosis and 1 month following completion of her adjuvant chemotherapy, she was hospitalized again for worsening of lower extremity weakness and pain. Repeat cervical spine MRI showed some worsening T2 signal change within the cord (thick white arrow) with no evidence of contrast enhancement. Acute therapy with plasma exchange and intravenous steroids were given over 5 days followed by rituximab induction (1,000 mg on days 0 and 14). Additional cancer screening with full body PET scan remained negative with clinical relapse.

resolution of contrast enhancement and improved T2 signal change (**Figure 2**).

Seven months after her initiation presentation she was readmitted to the hospital for worsening pain and weakness in the lower extremities. Neurologic exam demonstrated increased weakness, primarily in the left leg and arm.

MRI showed longitudinally extensive T2 signal abnormality within medial cord from C2 through T1 with no accompanying gadolinium enhancement. Repeat PET-CT showed no evidence of FDG-avid malignancy. Following PLEX and IVMP over 5 days, there was improvement in her strength. Given her clinical relapse, rituximab was initiated with induction dose at 1,000 mg on days 0 and 14 and the patient initiated a 4-week course of radiation therapy. She has since completed two cycles of rituximab infusions. Her oncologist continues to monitor for cancer, and her surveillance screen remains negative.

Fourteen months after initial visit, neurological examination significantly improved with one to two grades higher on all strength testing and she was able to ambulate with assistance using her walker, although she still must rely on wheelchair the majority of the time. Repeat CRMP5 antibody titer was now negative.

From the patient's perspective, she has had significant benefit after PLEX and chemotherapy but felt the discontinuation of chemotherapy had brought back some of her ongoing neuropathic pain symptoms and weakness. With the initiation of rituximab, most of the symptoms have subsided; however, mobility remains a challenge, but this is much better than her initial presentation. Her only ongoing bothersome symptom is lower back pain, potentially multifactorial involving spasms, degenerative disks, and neuropathic pain.

Neuronal Marker Results

See **Supplementary Material** for summary of neuronal marker results for each time point including **Supplementary Table 2** (0 months), **Supplementary Table 3** (6 months), and **Supplementary Table 4** (14 months).

0 Months (All Samples Collected Prior to Intervention With Empiric Immune Therapies)

Blood and spinal fluid collected at initial presentation showed significant elevation of all aforementioned biomarkers (see **Supplementary Table 1** for normal control values). Most notable increase was in the CSF. The universal axonal injury biomarker NfL CSF level 3,399.7 (control median: 1,241 pg/ml) and plasma was 782.62 pg/ml. Concomitantly elevated CSF levels of GFAP were observed in the CSF 35,402.8 (control median: 14,624 pg/ml) and plasma elevated at 283.32 pg/ml. CSF levels of UCH-L1 elevated at 8,932.28 pg/ml (control median: 989 pg/ml) and plasma was 240.58 pg/ml (no normal control data). Additionally, tau CSF elevated to 160.77 pg/ml (control median: 118 pg/ml) and plasma at 5.54 pg/ml (control median: 2.21 pg/ml).

6 Months

NfL plasma levels remained elevated but significantly reduced at 103.27 pg/mL. GFAP was 211.58 pg/mL, UCH-L1 15.34, and tau normalized at 0.73 pg/ml.

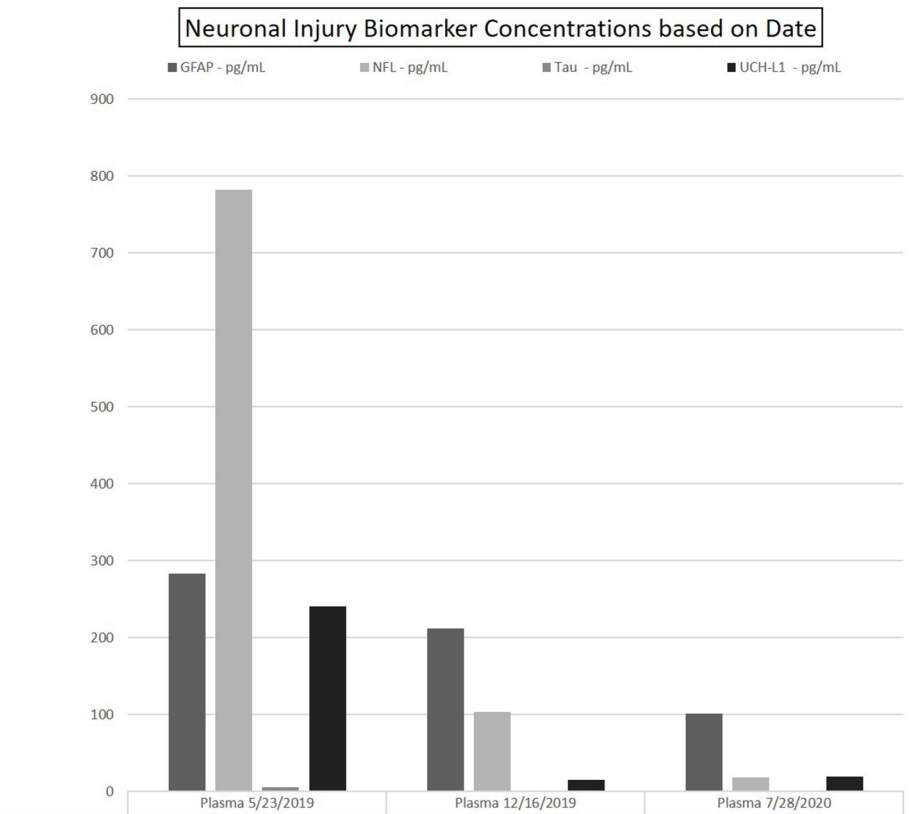
14 Months

Plasma NfL levels reduced to near normalization of 18.40 pg/mL. GFAP was 100.61 pg/mL, UCH-L1 19.53, and tau normalized at 1.12 pg/ml. See trend in **Figure 3**.

DISCUSSION

To our knowledge, this is the first case report of CRMP5-associated TM with serial collection of NfL and other CNS-derived proteins in which concentrations tended to correlate with clinical symptoms. Here, we provided an observation of four novel quantitative biomarkers in this patient's 14-month clinical course.

We noted an elevation of 2,640% of CSF concentrations of NfL over normative control values, potentially reflecting disease activity. Prior studies of inflammatory demyelinating diseases noted increased CSF NfL concentrations across various groups including clinically isolated syndrome (CIS; mean NfL: 15,138.50 pg/ml), NMOSD (mean: 15,400.00 pg/ml), and MS (mean: 5,789.50 pg/mL) (20). This study suggests no statistically significant association with classical biomarkers



	Plasma 5/23/2019	Plasma 12/16/2019	Plasma 7/28/2020
GFAP - pg/mL	283.3262355	211.580871	100.6122075
NFL - pg/mL	782.621897	103.270857	18.395885
Tau - pg/mL	5.541122	0.7398185	1.116042
UCH-L1 - pg/mL	240.5845395	15.347502	19.5396545

FIGURE 3 | Data depicting the fitted concentration values for GFAP, NFL, Tau, and UCH-L1 at time points 0–6–14 months. Date of sample collection utilized to represent these time points. Each run yielded 2-well readings on the N4PA; the averaged of each shown for clarity. CSF omitted from table due to exceedingly high concentrations of NfL as compared with the values in the patients' blood.

(OCB+/– in CIS and MS or AQP4 in NMOSD) and NfL concentrations but a potential relationship between CSF NfL level and disease activity represented by MRI contrast-enhancing lesions (20). Analogous associations in axonal injury biomarkers and imaging for traumatic brain injury (TBI) patients has also indicated potential clinical significance. Plasma GFAP concentrations may complement MRI as a biomarker for intracranial lesions undetectable on CT with the possible adjunct providing differentiation to traumatic pathology based on relative differences in GFAP concentration (21). Similar trends appear in our case of CRMP5-associated TM and MRI lesion enhancement accompanied with elevated neuronal biomarkers suggestive of the potential correlation. Blood-brain barrier (BBB) leakage, also delineated by enhancement on MRI, may be important for the detection of these biomarkers in the plasma.

In addition to NfL, our patient had an initial CSF GFAP concentration of 142% above normative control values. GFAP is considered to reflect the sum of degraded astrocytes in CSF and associations with NfL levels may denote clinical outcomes and/or relapse in MS (9, 22) and NMOSD (6). Additionally, there was an 803% increase in UCH-L1 and a mild elevation of tau. Initial plasma concentrations were elevated for all four biomarkers; this increase in blood concentrations were congruent with her elevations in CSF.

Markers of neuronal damage may potentially aid clinicians in predictions of patient outcome, nonrecovery deficits, and treatment efficacies. Studies in relapsing remitting MS (RRMS) have shown that NfL is most elevated during an attack, and as duration from attack increases, NfL decrease (23). In this case, there was drastic improvement in her neurologic exam and MRI after PLEX and steroids alone. This rapid improvement suggests early and aggressive intervention may ensure the best possible clinic outcome. Furthermore, this may suggest that early treatment could offer the least amount of neuronal damage from CRMP-5-associated TM as her markers of neuronal damage were significantly elevated at the time of her diagnosis and subsequently decreased over time. After discontinuation of chemotherapy, symptomatic relapse occurred 6 months after initial presentation. Neurologic exam indicated improvements

compared with initial presentation; however, during physical therapy, there was notable decreased stamina and fatigue. Repeat MRI showed no enhancement (**Figure 2D**) and PET/CT was negative for malignancy. Additionally, as shown in **Figure 3**, plasma NfL, GFAP, and UCH-L1 levels were greatly reduced, yet remained elevated compared with normative values. Plasma UCH-L1 dramatically reduced from 240.58 to 15.34 pg/ml and tau normalized. Interestingly, plasma NfL dramatically decreased while there was only a modest reduction in GFAP. Currently, GFAP evaluation as a marker in gauging TBI has indicated higher initial GFAP with a biphasic release in serum with level decreasing during the first 6 months but increasing over subsequent visits postinjury, which may correspond to our patient and her maintaining elevated levels (24). Previous MS studies evaluating GFAP as a biomarker in disease progression indicated augmented GFAP levels in MS patients, noting strong correlation between GFAP in CSF and neurological disability during MS progression (9). After adjusting for age, GFAP and NfL increased compared with healthy controls but GFAP was only statistically significant from examinations 8–10 years apart indicating the potential use GFAP in the neurodegenerative process (9). Therefore, continuous monitoring of NfL and GFAP in tandem may provide further indications of not only initially sustained damage and treatment effectiveness but disease progression in CRMP5-associated TM patients.

Additionally, as the 6-month sample collection was conducted during a clinical relapse, this NfL reduction of 86.80% stipulates that neuronal damage severity may have been hindered with acute immunotherapies and treatment of her underlying malignancy. Moreover, given that her relapse occurred upon discontinuation of chemotherapy, a potential for degree of biomarker elevation might provide a prognostic indicator toward the necessity of long-term immunosuppression.

Following treatment with rituximab, at 14 months past initial time point, the patient showed significant clinical improvements and improved strength. Plasma NfL and GFAP at this point were only mildly elevated and the reduced plasma concentration correlated with neurologic exam and radiographic improvements. This observed correlation suggests monitoring these biomarkers may be useful to quantify disease severity, progression, and treatment efficacy. Notably, plasma UCH-L1 remained elevated with a slight increase to 19.38 pg/ml (from 15.35 at 6 months), albeit dramatically decreased from initial presentation (240.58 pg/ml).

Our observed drastic elevation of neuronal injury biomarkers at disease onset might further indicate damage intensity due to mechanisms of action and inflammatory response in this case. The autoimmune response to intracellular antigens, like CRMP5, may cause damage indirectly by immune complex formation, immune activation, and other processes (25). However, the role of antibodies causing neuronal injury remains controversial. Antibodies against intracellular targets are not thought to be directly pathogenic, and rather, likely mediate damage *via* cytotoxic T cells; although, there has been some evidence to dispute this assumption (26, 27). PNDs associate with intracellular antibodies are often characterized by irreversible, neuronal death and can be refractory to immune therapy. Larger, longitudinal prospective studies of neuronal injury biomarkers in CRMP5 autoimmunity and other PNDs may provide some insight in our understanding of disease pathogenesis and approaches to treatment.

CONCLUSION

This longitudinal analysis of neuronal injury biomarkers in CRMP5-associated TM provides a case example of the possible utility of these biomarker with regard to disease activity and treatment response in PNDs. We observed neurologic improvements and the absence of contrast enhancement on subsequent MRIs that correlated with the reduction of NfL and GFAP concentrations. Similar trends have been reported in other neurological diseases. Further understanding is required regarding the relationship of these neuronal markers and their association with neurologic outcomes and treatment response, yet the speculative mechanisms proposed may provide insight for future research. A strong need for randomized, controlled clinical trials to evaluate the efficacy of immunotherapy in autoimmune and paraneoplastic neurological disease exists. Using neuronal injury biomarkers may provide a valuable outcome-surrogate marker and should be explored to assist in the development of future clinical trials.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was supported by the Rocky Mountain MS Center and the Drake family in the name of Susan Drake.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.691509/full#supplementary-material

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Conflict of Interest: JB reports personal fees from Roche, personal fees from Genentech, personal fees from Viela Bio, personal fees from Chugai Pharma, personal fees from Alexion, grants and personal fees from Novartis, personal fees from Genzyme, personal fees from Teva Neuroscience, grants and personal fees from EMD Serono, personal fees from Frequency Therapeutics, personal fees from Equillium, personal fees from Clene Nanoscience, personal fees from Mitsubishi-Tanabe, personal fees from Reistone Bio, grants from National Institutes of Health, grants from Guthy Jackson Charitable Foundation, and grants from National Multiple Sclerosis Foundation, outside the submitted work. In addition, JB has a patent for aquaporumab issued. TV has received compensation for lectures and consultancy from Biogen, Genentech/Roche, Siranax, Celgene, EMD Serono, and Novartis and has received research support from Rocky Mountain Multiple Sclerosis Center, Celgene, Biogen, Anokion, Genentech, F. Hoffmann-La Roche Ltd, GW Pharma, and TG Therapeutics, Inc. AP has received research funding from the University of Colorado and Rocky Mountain MS Center, consulting fees from Genentech/Roche and Alexion, and honorarium from MedLink and publication royalties from Springer.

The remaining 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.

frontiers in Neurology

MINI REVIEW
published: 18 August 2021
doi: 10.3389/fneur.2021.683048

Update in Autoimmune Movement Disorders: Newly Described Antigen Targets in Autoimmune and Paraneoplastic Cerebellar Ataxia

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Movement disorders are a common feature of many antibody-associated neurological disorders. In fact, cerebellar ataxia is one of the most common manifestations of autoimmune neurological diseases. Some of the first autoantibodies identified against antigen targets include anti-neuronal nuclear antibody type 1 (ANNA-1 or anti-Hu) and Purkinje cell cytoplasmic antibody (PCA-1) also known as anti-Yo have been identified in paraneoplastic cerebellar degeneration. Historically these antibodies have been associated with an underlying malignancy; however, recently discovered antibodies can occur in the absence of cancer as well, resulting in the clinical syndrome of autoimmune cerebellar ataxia. The pace of discovery of new antibodies associated with autoimmune or paraneoplastic cerebellar ataxia has increased rapidly over the last few years, and pathogenesis and potential treatment options remains to be explored. Here we will review the literature on recently discovered antibodies associated with autoimmune and paraneoplastic cerebellar ataxia including adaptor protein-3B2 (AP3B2); inositol 1,4,5-trisphophate receptor type 1 (ITPR1); tripartite motif-containing (TRIM) proteins 9, 67, and 46; neurochondrin; neuronal intermediate filament light chain (NIF); septin 5; metabotropic glutamate receptor 2 (mGluR2); seizure-related 6 homolog like 2 (SEZ6L2) and homer-3 antibodies. We will review their clinical characteristics, imaging and CSF findings and treatment response. In addition, we will discuss two clinical case examples of autoimmune cerebellar ataxia.

Keywords: autoimmune cerebellar ataxia, paraneoplastic cerebellar ataxia, AP3B2 antibody, ITPR1 antibody, TRIM9 antibody, TRIM67 antibody, TRIM46 antibody, mGluR2 antibody

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

Received: *19 March 2021*
Accepted: *28 July 2021*
Published: *18 August 2021*

Citation:
Garza M and Piquet AL (2021) Update in Autoimmune Movement Disorders: Newly Described Antigen Targets in Autoimmune and Paraneoplastic Cerebellar Ataxia. Front. Neurol. 12:683048. doi: 10.3389/fneur.2021.683048

INTRODUCTION

Cerebellar ataxia has a broad differential diagnosis including both acquired and genetic causes, however autoimmune etiologies or paraneoplastic cerebellar degeneration (PCD) should be considered in most case, particularly in adults (1). The first autoantibodies to be identified against neuronal targets include anti-neuronal nuclear antibody type 1 (Anti-Hu) in 1965 (2) and Purkinje cell cytoplasmic antibody (anti-Yo) in 1983 (3) which both represent classic paraneoplastic neurological syndromes. It is hypothesized that cross-reactivity between proteins expressed on the tumor and neuronal antigens is responsible for the development of neurologic symptoms, of which cerebellar ataxia is one of the most common manifestations (4). Since those two autoantibodies were described, the rate of discovery of new autoantibodies to other neuronal targets has been

rapidly increasing and there are now several antibodies known to cause cerebellar ataxia both in the setting of malignancy (i.e. paraneoplastic) and in its absence (i.e. idiopathic autoimmune).

Paraneoplastic cerebellar degeneration is a diagnosis under the umbrella of immune-mediated cerebellar ataxias (IMCAs), which also includes post-infectious cerebellitis and gluten ataxia (5). Patients who present with subacute onset cerebellar ataxia but in the absence of malignancy or a known pathogenic antibody are now deemed to have primary autoimmune cerebellar ataxia (PACA) (6). This is in contrast to neuronal antibodies that have already been shown to be directly involved in the pathogenesis of ataxia (e.g. dipeptidyl-peptidase-like-6 [DPPX], metabotropic glutamate receptor 1 [mGluR1], glutamic acid decarboxylase [GAD]-65). See **Table 1** for clinical criteria for PACA. These patients tend to present with a subacute onset of gait and limb ataxia as well as dysarthria or nystagmus and they may have objective findings such as inflammatory cerebrospinal fluid (CSF) or magnetic resonance imaging (MRI) showing abnormalities in cerebellum. Patients with suspected PACA should receive immunotherapy trials such as steroids, intravenous immunoglobulins (IVIg) or plasmapheresis (PLEX), although the likelihood of improvement seems to depend on whether the antibody target is a cell surface or an intracellular antigen (5, 6).

The aim of this review article is to focus on recently discovered autoantibodies against neuronal targets identified in syndromes cerebellar ataxia and includes two clinical case examples of adaptor protein-3B2 (AP3B2) and tripartite motif-containing (TRIM)-46. In these the majority cases, an associated antibody has been identified, but it is not yet clear whether there is a direct pathogenic effect with the exception of Metabotropic glutamate receptor-2 (mGluR2).

Table 2 includes an expanded summary on recently described autoantibodies including seen in PACA as well as prominent extracerebellar phenotypes (e.g. Caspr2).

TABLE 1 | Criteria for the diagnosis of PACA.

1. Predominantly subacute or acute pure cerebellar syndrome (gait ataxia that may be associated with variable degrees of limb incoordination, dysarthria, nystagmus, diplopia)
2. MRI at presentation usually normal or may show primarily cerebellar vermian atrophy with (if available) reduced MR spectroscopy (NAA/Cr ratio) of the vermis
3. At least 2 of the following:

a. CSF pleocytosis and/or positive CSF-restricted IgG oligoclonal bands
b. History of other autoimmune disorders or family history of autoimmune disorders in first-degree relatives
c. Presence of antibodies that support autoimmunity but not yet shown to be either directly involved in ataxia pathogenesis which includes autoantibodies associated with non-neurological autoimmune disease* or autoantibodies reported in only a few patients with ataxia (therefore significance is less well characterized but raises suspicion of PACA)**, or to be markers of ataxia with a known trigger***

4. Exclusion of alternative causes made by an experienced neurologist or ataxia specialist (including other causes of immune ataxia such as PCD, GA, PIC and ones that are associated with well-characterized pathogenic antibodies)

*Examples include thyroid peroxidase, thyroglobulin, anti-SSA (Ro) and anti-SSB (La). **Examples include anti-ITPR1, anti-Homer-3, anti-AP3B2, anti-neurochondrin, anti-Septin-5, anti-MAG. ***Examples include anti-Yo in paraneoplastic cerebellar degeneration or antigliadin in gluten ataxia. Adopted from: (6). CSF, cerebrospinal fluid; GA, gluten ataxia; MRI, magnetic resonance imaging; NAA/Cr, N-Acetylaspartate/Creatine; PACA, primary autoimmune cerebellar ataxia; PCD, paraneoplastic cerebellar degeneration; PIC, post-infectious cerebellitis.*

METHODS

This review focuses on newly described antibodies in the literature seen in PACA and antibody syndromes in which are newly commercially available for testing – thus our understanding of these antibodies will continue to evolve and these syndrome will likely become more readily recognized. We performed a systematic literature search in PubMed to identify autoantibodies reported against neuronal targets in autoimmune and paraneoplastic cerebellar ataxia after 2014. We also included antibody syndromes that are more recently commercially available in the United States for testing since 2021 (35, 36).

Adaptor Protein-3B2 (AP3B2)

β-neuronal adaptin-like protein, located in neuronal cytoplasm, mediates synaptic vesicle coat assembly. Darnell and colleagues first identified it as an antigen target in 1991 in a 32-year-old woman with subacute progressive ataxia (5). At the time, this was the only known case, but more recently, Honorat and colleagues identified a series of patients with this novel but identical sera and CSF immunofluorescence assay (IFA) staining patterns on mouse nervous system tissue (7). This antigen was identified as β-neuronal adaptin-like protein, now named adaptor protein-3B2 (AP2B2).

All patients reported presented clinically with subacute progressive gait disturbances, including both cerebellar and sensory ataxias. These clinical presentations coincide with the areas of the nervous system that were shown to have greatest staining by IFA; namely the cerebellar Purkinje cells, the dorsal spinal column and dorsal root ganglia. Malignancy in general did not seem to be a precipitant of this autoimmune cerebellar ataxia, with only one patient out of nine found to have an underlying cancer (renal cell carcinoma) (7).

In terms of objective data, CSF was abnormal in all patients. Slight pleocytosis was present in a few patients (median WBC count 7/μL) and others had elevated protein (median 46mg/dL) and increased immunoglobulin-G (IgG) index or oligoclonal bands (7). In the patients who presented with ataxia, brain MRIs showed cerebellar atrophy. Unfortunately, patients treated with immunotherapies did not report improvement, and some were noted to have progressive symptoms despite treatment (7).

Given its intracellular location, AP3B2 antibody is unlikely pathogenic, rather simply a biomarker for CD8+ cytotoxic T cell-mediated damage.

Clinical Case Vignette #1

A 15-year-old female with no significant past medical history presented with progressive left sided weakness and ataxia. One month prior, she noticed her left hand became clumsy, her handwriting became illegible (she is left-handed) and she had

TABLE 2 | Summary table of autoantibodies against neuronal targets identified in syndromes of cerebellar ataxia.

Antibody	Average age (range) in years	Clinical presentations	Diagnostic testing	Response to immunotherapy	Association with cancer	Antigen target location/ antibody testing method	References
AP3B2	39 (24–58)	Cerebellar ataxia, Peripheral neuropathy	CSF: pleocytosis, elevated protein, and/or increased IgG index and OCB MRI: cerebellar atrophy	Poor	1/ 9 patients had cancer (RCC)	Intracellular/ IFA staining in cerebellum spinal cord gray matter, dorsal root ganglia and sympathetic ganglia; confirmed with WB and CBA	(7)
Caspr2	50s (19–80); reported in pediatric case series with mean age of 13 (2–17 years)	Encephalitis, Cerebellar ataxia, Morvan Syndrome, peripheral nerve hyperexcitability, neuromyotonia	CSF- About 25% with abnormalities including pleocytosis and/or elevated protein, rarely OCB MRI- About half abnormal with T2 hyperintensities in temporal lobes or hippocampal/mesial temporal atrophy	Positive	SCLC, Thymoma	Extracellular/ IFA; confirmed on CBA*	(8–12)
GFAP	42 (21–73)	Meningoencephalitis, myelitis, ataxia and other movement disorders	CSF- Almost all patients with pleocytosis, elevated protein, half with OCB MRI- Abnormal with T2 hyperintensities or contrast enhancement	Positive	Thymoma in about ¼, other rare associations (breast, ovarian, GI, prostate, melanoma, parotid adenoma, teratoma)	Intracellular/ IFA with cytoplasmic filament staining restricted to astrocyte populations; confirmed on CBA*	(13–16)
Homr 3	Only three case described	Cerebellar ataxia Encephalitis	CSF- pleocytosis, elevated IgG index in one patient MRI- Variable, can be normal or have cerebellar atrophy	Variable	1 SCLC identified, otherwise none	Intracellular	(17–19)
IgLON5	64 (46–83)	Sleep disorder, bulbar symptoms, gait abnormalities, and oculomotor difficulties	CSF- half normal and other half with elevated protein, pleocytosis or OCB MRI- normal in most patients; a subset with mild brainstem and cerebellar atrophy	Poor	None	Extracellular/ IFA; confirmed on CBA*	(20)
ITPR1	64 (7–83)	Cerebellar ataxia, Peripheral Neuropathy, Encephalitis, myelopathy	CSF: pleocytosis and/or elevated protein MRI: Varies, can be normal or have T2 signal changes in various locations, cerebellar atrophy also seen	Poor	5/14 patients had cancer (3 breast, 1 RCC, 1 endometrial)	Intracellular/ IFA staining in cerebellum in a "Medusa head-like" cytoplasmic staining pattern; confirmed with CBA*	(21, 22)
KLHL11	28.5 (9–76)	Brainstem and cerebellar syndrome, encephalitis	CSF- pleocytosis, elevated protein, increased IgG index and/or OCBs MRI- Mostly abnormal with cerebellar atrophy or midbrain/cerebellar T2 hyperintensities	Variable	Teratomas, Testicular seminoma, mixed germ cell tumors	Intracellular/ IFA with robust reactivity with cytoplasm of large neurons in brainstem and deep cerebellar nuclei; confirmed by CBA	(23, 24)
mGluR2	Only 2 patients, 78yo F and 3yo F	Cerebellar ataxia	CSF only obtained on one patient and was normal MRI- one with increased T2 signal in cerebellum, other with enhancement of cerebellum	Unclear (1 positive, 1 poor)	Small cell tumor of unknown origin and alveolar rhabdo-myosarcoma	Extracellular/ IFA with staining in the cerebellum limited to granular cell layer and hippocampus; confirmed with CBA	(25)

(Continued)

TABLE 2 | Continued

Antibody	Average age (range) in years	Clinical presentations	Diagnostic testing	Response to immunotherapy	Association with cancer	Antigen target location/ antibody testing method	References
NIF	65 (47–87)	Cerebellar ataxia, encephalopathy, cranial neuropathies	CSF- pleocytosis, elevated protein and/or OCB MRI- Variable, some normal, some with T2 signal or enhancement	Positive	Neuro-endocrine tumors	Intracellular/ IFA with intense staining of neuronal cytoplasmic filaments in CNS and cerebellar granular layer and peri-Purkinje cell regions; confirmed with CBA*	(26)
Neuro chondrin	27 (2–67)	Cerebellar ataxia	CSF- all with pleocytosis or OCB MRI- cerebellar and supratentorial gray matter atrophy	Positive	No malignancies identified	Intracellular/ IFA with synaptic-type hippocampal staining pattern; confirmed with WB	(27, 28)
Septin 5	59 (47–62)	Cerebellar ataxia, oscillopsia	CSF- only 1 patient with data available, elevated protein MRI- one normal, one with cerebellar atrophy	Variable	No malignancies identified	Intracellular	(29)
SEZ6L2	62 (54–69)	Cerebellar ataxia, extrapyramidal symptoms	CSF- pleocytosis in 1 patient, others normal MRI- Cerebellar atrophy	Poor	1 patient with ovarian cancer diagnosed 4 years later	Extracellular/ IFA with intense staining of neuropil of hippocampus and molecular layer and synaptic buttons of granular layer of cerebellum; confirmed with CBA	(30–32)
TRIM 46, 9 & 67	71 (64–78)	46-Cerebellar ataxia, 9 & 67- Cerebellar ataxia, RPD, encephalomyelitis	CSF- pleocytosis, elevated protein, OCB MRI- largely normal	Poor	SCLC	Intracellular/ IFA; confirmed with CBA	(33, 34)

**Antibody commercially available for testing. AP3B2, Adaptor Protein-3B2; Caspr2, contactin-associate protein-2; CBA, cell-based assay; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; IFA, tissue-based indirect immunofluorescence assay; IgG, immunoglobulin G; ITPR1, Inositol 1,4,5-trisphosphate Receptor Type 1; KLHL11, Kelch-like protein 11; MRI, magnetic resonance imaging; mGluR2, metabotropic glutamate receptor 2; NIF, neuronal intermediate filament light chain OCB, oligoclonal bands; RCC, renal cell cancer; RPD, rapidly progressive dementia; SCLC, small cell lung cancer; SEZ6L2, seizure-related 6 homolog like 2; TRIM, tripartite motif-containing; WB, western blotting assay.*

difficulty completing fine motor tasks such as painting her nails. She also developed a feeling of "heaviness" in her left leg and was unable to keep up with her teammates at practice. With the progression of her symptoms including worsening gait, she was referred to see a neurologist.

On exam, the patient demonstrated focal left-sided abnormalities including dysmetria with finger-to-nose and heel to shin, dysrhythmia of finger taps and difficulty with rapid alternating movements. Cranial nerves were all intact and there was no dysarthria or truncal ataxia. Brain MRI brain showed mild left cerebellar atrophy as well as a 6 mm T2 hyperintense lesion to the left of the cerebellar vermis (**Figure 1**). Lumbar puncture revealed inflammatory CSF with WBC 23 (lymphocytic predominance) and 13 unique oligoclonal bands. An extensive infectious workup was unrevealing including Next-Generation sequencing (NGS) (UCSF center for Next-Gen precision for diagnostics). She was started on empiric IV methylprednisolone for treatment of a presumed autoimmune disorder. Positron emission tomography and computed tomography (PET-CT) scan was negative for malignancy, although notably three weeks prior to the onset her neurologic symptoms she did have a pilomatrixoma removed from her neck, which was of unclear significance. Autoantibody testing on her CSF performed at Mayo Clinic Laboratories was positive for an unclassified antibody with synaptic antibody features.

The patient was discharged home after treatment with IV methylprednisolone and was subsequently treated with weekly IV methylprednisolone 1g (as well as one 3 day course of IVIg which she didn't tolerate due to headache) before being started on rituximab at 1,000 mg day 0 and day 14. She responded well to rituximab therapy and has had moderate improvement in coordination on the left and improvement in her gait. She received rituximab therapy over the course of one year and has since remained off immunotherapy with clinical and radiographic follow up over three years. Repeat MRIs have been stable in terms of degree of atrophy in left cerebellum and size of

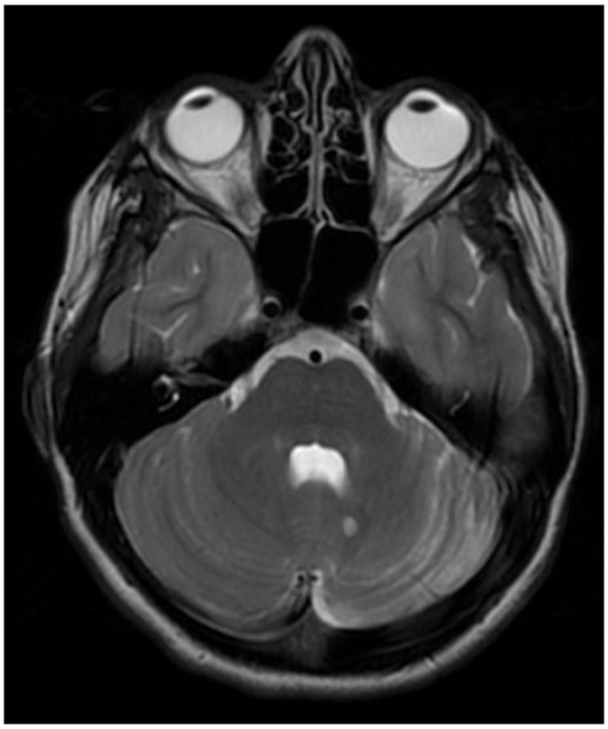

FIGURE 1 | T2 sequence of brain MRI demonstrating hemi-atrophy of the left cerebellum in addition to a 6 mm T2-hyperintense lesion near the cerebellar vermis on the left. Additional brain imaging not show any further T2/FLAIR (fluid-attenuated inversion recovery) abnormalities in the cerebellum, cerebrum or corpus callosum (sequences not shown).

the T2 hyperintense lesion. She is screened yearly for malignancy, which has been negative over the course of three years. Two years after her diagnosis of autoimmune cerebellar ataxia, her CSF evaluation at Mayo Clinic Laboratories revealed a positive AP3B2 antibody. Two unique features seen in this case, not reported in the prior literature, include the hemi-cerebellar atrophy and the positive response to immune therapy.

Inositol 1,4,5-Trisphosphate Receptor Type 1 (ITPR1)

Inositol 1,4,5-trisphosphate receptor type 1 (ITPR1) is an intracellular ligand-gated calcium channel mainly expressed in membranes surrounding the endoplasmic reticulum. It was first identified as an antibody associated with autoimmune cerebellar ataxia in 2014 by Jarius and colleagues after immunohistochemical testing showed binding of IgG1 antibodies in molecular and Purkinje cell layers on animal cerebellum sections in a pattern similar to, but not matching that of, anti-Ca/anti-RhoGTPase-activating protein 26 (ARHGAP26) antibodies (21, 37).

The largest cohort of patients described with ITPR1-IgG antibodies included five who presented with cerebellar ataxia at an average age of 64 (22). Of the five patients with CSF available, four had abnormalities such as pleocytosis or elevated protein. About half of the patients with ITPR1 IgG antibodies had an underlying malignancy including three breast and one renal carcinoma. Immunotherapy response in these patients is reported to be poor with all ten patients who received immunotherapies in this review failing to improve. Interestingly, there was one case report of a 31-year-old woman with a three-year history of slowly progressive cerebellar ataxia found to have ITPR1 antibodies and BRCA1 mutation. Serial malignancy screenings identified a ductal carcinoma 6 years later, which also had significant ITPR1 expression (38).

The fact that ITPR1 antibodies express such high affinity for Purkinje cells in patients who present with cerebellar ataxia and with such high titers suggests that these antibodies could be pathogenic (37). Additionally, ITPR1 mutations are known to be associated with spinocerebellar ataxias. More studies needed to determine whether ITPR1 may indeed be pathogenic.

Tripartite Motif-Containing (TRIM) Proteins 9, 67, and 46

Tripartite motif (TRIM) containing proteins are part of a large group of E3 ubiquitin ligases involved in many different processes such as cellular signaling, carcinogenesis and immunity. TRIM 9 and TRIM 67 have a large role in neuronal development and axonal growth and are highly concentrated in Purkinje cells both in the hippocampus and cerebellum (33). TRIM 46, expressed more diffusely in the central and peripheral nervous system, plays a role in axon growth (34). All three of these specific TRIM proteins have been described as antigen targets in a handful of patients presenting with paraneoplastic cerebellar ataxia.

The few patients described with TRIM9 and TRIM67 have all presented with a subacute onset of severe cerebellar ataxia (33). Presentations with TRIM 46 antibodies seem to be more varied (which could be explained by more diffuse expression of TRIM 46 in the CNS) and has included encephalitis and rapidly progressive dementia, along with cerebellar ataxia (34). Brain imaging is reported as normal in most of these patients or if abnormal may demonstrate some cerebellar atrophy. CSF is often inflammatory with pleocytosis, elevated protein or the presence of oligoclonal bands. These antibodies are strongly associated with malignancy, specifically small-cell lung cancer (33, 34). In the report of two patients with anti-TRIM9 and anti-TRIM67 antibodies, one patient treated with immunotherapy did not show any improvement, and one other patient who had regression of his cancer continued to have severe ataxia years down the line (33). This finding suggests that TRIM9 and TRIM67 are biomarkers of rare cases of paraneoplastic cerebellar ataxia and prompt diagnosis is necessary to try to initiate early immunotherapy, as the mechanism of damage is likely related to CD8+ T cells that seems to cause irreversible neuronal death (33).

Clinical Case Vignette #2

A 63-year-old woman with a one-year history of metastatic endometrial cancer was recently started on pembrolizumab and lenvatinib for treatment of her cancer. Seven months after initiation of pembrolizumab she developed neurological symptoms of vertigo and ataxia. Pembrolizumab was

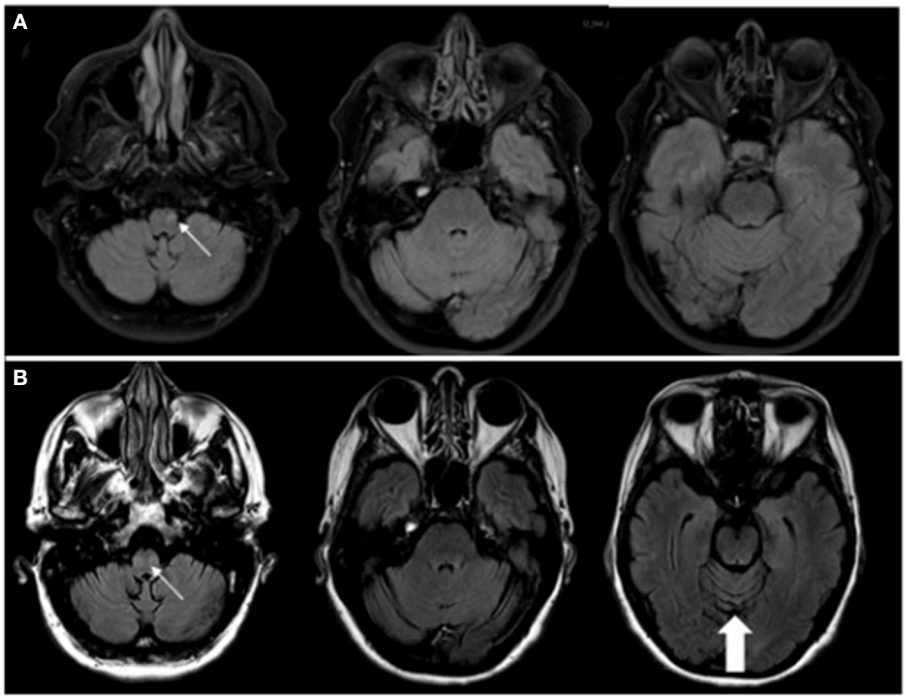

FIGURE 2 | (A) MRI sequences of T2/FLAIR (fluid-attenuated inversion recovery) with STIR (short tau inversion recovery; i.e. fat suppression) at the onset of neurological symptoms of vertigo and ataxia demonstrating a small T2, non-enhancing lesion (contrast imaging not shown) in the left medulla (thin, white arrow). **(B)** with T2/FLAIR MRI 3 months later demonstrating T2 changes seen bilaterally within the medulla (thin, white arrow) with no associated contrast enhancement. There is noted cerebellar atrophy, particularly seen in the superior cerebellum (thick, white arrow), compared to the MRI from 3 months prior.

discontinued and she was started on a short course of high-dose oral steroids with no response in her neurological symptoms. She continued to progress with worsening mobility, gait imbalance, diplopia and vertigo with nausea and vomiting so she was referred to Neurology for further evaluation. Brain MRI at the time of her symptom onset was only remarkable for a small T2 lesion the left medulla and no evidence of cerebellar atrophy. Repeat brain MRI at the time of her neurological evaluation revealed subsequent cerebellar atrophy, worsening T2 signal change in the medulla bilaterally (**Figure 2**), and new T2/FLAIR signal change in the left thalamus. CSF analysis revealed 0 WBCs, mildly elevated protein at 67 mg/dL (ref < 45 mg/dL), 6 unique oligoclonal bands, elevated IgG index of 1.27 (ref 0.28–0.66 ratio), and an unclassified antibody identified on IFA screen at Mayo Clinic Laboratories. Subsequent research antibody testing revealed a positive TRIM46 antibody confirmed on cell-based assay (CBA). Novel autoantibody testing completed on a research basis at UCSF laboratories also confirmed the presence of TRIM46-IgG (**Figure 3**). The patient was subsequently diagnosed with anti-TRIM46 antibody-associated cerebellar ataxia with suspected paraneoplastic etiology vs. pembrolizumab-associated autoimmune cerebellar ataxia. Notably, immune checkpoint inhibitors (ICIs) can cause a variety of clinical phenotypes and various underlying disease mechanisms have been observed. When classic paraneoplastic associations are seen (i.e. classic phenotypes such as cerebellar ataxia with clear antibody profiles associated with cancer), this could potentially be augmented by the anticancer immune response against onconeural antigens (39). This patient will remain off her pembrolizumab given this complication. She received acute treatment with 5 days of IV methylprednisolone and plasma exchange followed by the initiation of monthly IV cyclophosphamide. Two months after her treatment with immune therapy, she demonstrated mild improvement in her mobility (she is able to walk and transfer with assistance after being wheelchair-bound) and improvement of her vertigo. However, at six months after completion of her cyclophosphamide course she remains severely disabled neurologically.

Neurochondrin

Neurochondrin is an intracellular protein expressed in neurons in a somato-dendritic distribution. In concert with G-protein-coupled receptors such as mGluR1 and mGluR5, it has been shown to have an important role in synaptic plasticity, particularly in the cerebellum (27).

There have been two small cohorts of patients described that were found to have anti-neurochondrin antibodies. They all presented with a rapidly progressive cerebellar ataxia, and a few demonstrated brainstem findings such as eye movement abnormalities or dysphagia. CSF had an inflammatory profile in all patients, including pleocytosis, elevated protein, oligoclonal bands and/or increased IgG index. While some MRIs were

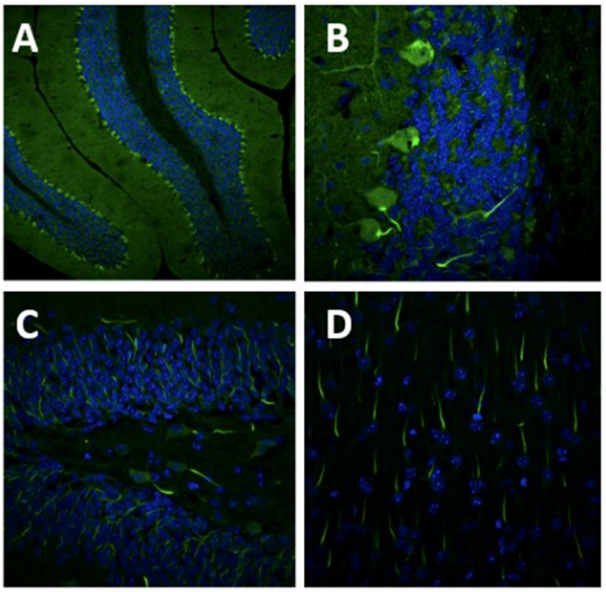

FIGURE 3 | Images of **(A)** 10x magnification and **(B)** 60x magnification of cerebellar tissue demonstrating antigen staining of the patient's Trim46 antibody (in green) and nuclei are in blue. **(C)** There are axon initial segments staining in green in the dentate gyrus and **(D)** in the cortex as well. *Images courtesy of Dr. Christopher Bartley and team at University of San Francisco (USCF) Weill Institute for Neurosciences laboratory*.

initially normal or showed increased T2/FLAIR signal (mainly in pons, midbrain, and hippocampus), almost all patients eventually developed significant cerebellar atrophy on subsequent MRI scans (27, 28). Malignancy was only identified in one patient (uterine carcinoma) (28). Unfortunately, none of the patients treated with immunotherapy showed any signs of improvement.

Given its intracellular expression, damage related to anti-neurochondrin IgG antibodies is likely mediated by a cytotoxic T-cell response.

Neuronal Intermediate Filament Light Chain

Neuronal intermediate filaments (NIF) are a group of proteins that are integral to the structure and function of neurons in the central and peripheral nervous systems. Neurofilament light chain proteins are a subtype of NIF proteins that have been implicated as antigen targets in patients with cerebellar ataxia and encephalopathy (26).

Subacute but rapidly progressive cerebellar ataxia as well as encephalopathy were the two most common clinical presentations in a large cohort of patients described in 2018 (26). Of the 9 patients with data available, 5 had an abnormal MRI consisting of cerebellar atrophy and/or T2 hyperintensities in patients with ataxia (26). Most patients had CSF abnormalities including lymphocytic pleocytosis (median 41.5 WBCs in patients with CA) and/or CSF-restricted oligoclonal bands and increased protein (median 116 mg/dL) (26). NIF-IgG was strongly associated with malignancy, with cancer in 77% of these patients, most commonly neuroendocrine tumors including small-cell lung cancer. Patients with NIF antibodies with reported immunotherapy treatment tended to have improvement of their neurologic symptoms, which is less common for paraneoplastic neurologic disorders mediated by antibodies to intracellular antigens (26).

Septin 5

Septins are a group of cytoskeletal GTP-binding proteins with many different functions; although in the CNS they seem to have an important role in synaptic vesicle formation and exocytosis (40). The anti-Septin 5 antibody was identified by Honorat and colleagues at Mayo Clinic when indirect IFA of patient's sera and CSF demonstrated a novel staining pattern of synaptic regions of mouse cerebrum and cerebellum (29).

Anti-septin 5 antibodies have been identified in only a handful of patients. Clinically these patients all presented with a rapidly progressive cerebellar syndrome, including two with oscillopsia. CSF was only available for one patient and was significant for increase IgG synthesis rate. MRI imaging was only available for two patients, one of which was normal and the other showing cerebellar atrophy. There were no malignancies identified in any of the patients. In terms of treatment, only two patients were treated with immunotherapies which resulted in significant improvement for one patient, but only transient improvement in the other patient who ended up dying 6 months later (29).

Although septin-5 is largely expressed intracellularly, it is revealed extracellularly during exocytosis. It is therefore unclear whether septin-5 antibodies directly pathogenic or if they mediate damage via cytotoxic T-cells.

Metabotropic Glutamate Receptor 2 (mGluR2)

Metabotropic glutamate receptors (mGluR) are a family of cell surface G-protein-coupled receptors that bind glutamate and therefore play a significant role in synaptic transmission and neuronal excitability. Metabotropic glutamate receptor 1 was the first to be identified in patients with cerebellar ataxia in the early 2000s. More recently in 2019, mGluR2 was described in two patients with subacute onset cerebellar ataxia (25). There are 8 subtypes of mGluRs divided into 3 subgroups: Group I includes mGluR1 and 5 in which both are autoimmune targets in cerebellar ataxia and encephalitis associated with Hodgkin lymphoma (41, 42); Group II comprises of mGluR2 and 3; and Group III includes the remaining subtypes. The main physiologic role of mGluR2 is to modulate glutamatergic and GABAergic synaptic transmission and mGluR2 antibodies could potentially alter these functions (25, 43). Immunohistochemical studies in rat cerebellar cortex demonstrated mGluR2 and 3 immunoreactivity in the cerebellar cortex localizing to the Golgi cells with the majority of the Golgi cells distributed mainly in the Purkinje cell layer and superficial part of the granular layer (44).

The two patients described with anti-mGluR2 antibodies are on opposite ends of the spectrum in terms of age; one patient was a 78-year-old woman and the other a 3-year-old girl. The older woman presented initially with intermittent episodes of ataxia that eventually became progressive and constant. The young girl developed ataxia and dysarthria after a 3-day prodrome of fever,

nausea and vomiting. Both patient's MRIs were abnormal with hyperintense T2 cerebellar lesions including patchy enhancement of cerebellum in the 3-year-old. CSF was unavailable for the older patient but was normal in the young girl. Both received immunotherapy with drastically different outcomes. The older woman had progressive symptoms unresponsive to therapies while the young girl had complete recovery after receiving IV methylprednisolone and IVIg. Malignancy was identified in both patients after the development of neurologic symptoms. Small-cell neuroendocrine cancer of unknown origin was discovered on inguinal node biopsy in the 78 year-old woman three years after symptom onset, and the young girl was found to have alveolar rhabdomyosarcoma one year later (25).

Unlike other paraneoplastic cerebellar ataxias described here, mGluR2 antibodies target an extracellular antigen. Like anti-mGluR1 antibodies, mGluR2 antibodies likely have a direct pathogenic effect.

Seizure-Related 6 Homolog Like 2 (SEZ6L2)

SEZ6L2 is highly expressed in the hippocampus and cerebellar cortex and is an auxiliary subunit of the α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor (30). SEZ6L2 modulates AMPA receptor function by binding to adducin and glutamate receptor 1 (45). In 2014, SEZ6L2 antibodies were identified in a patient with subacute cerebellar ataxia and retinopathy, and later in another patient with cerebellar ataxia associated with hypomimia, bradykinesia, and postural instability (30–32). Since then, Landa and colleagues described four more cases in 2020 with SEZ6L2 antibodies identified in serum and CSF by immunohistochemistry on rate brain sections and immunoprecipitation from rat cerebellar neurons; all patients were then identified on CBA with unclassified neuropil antibodies (32). The median age of these four patients was 62 years old and patients presented with a subacute gait ataxia, dysarthria and mild extrapyramidal symptoms (32). Only one out of four patients had a CSF pleocytosis and three patients had evidence of moderate cerebellar atrophy with no evidence of contrast enhancement or other inflammatory features (32). In these series, none of the patients improved with immunotherapy (32).

Homer-3

Homer-3 is expressed at a high level in Purkinje cells (17, 37). Anti-Homer-3 antibody-associated cerebellar ataxia is rare and only reported in three cases (17–19, 37). The first case was presented in a 65-year-old female with vertigo, vomiting, dysarthria and severe subacute limb and gait ataxia (18). A second case was then reported in a 38-year-old man with a pancerebellar syndrome followed by encephalitis, seizures, and papilledema (19). Later a third case was described in a 51-year-old women with a pancerebellar syndrome (17). CSF analysis was abnormal in all cases including lymphocytic pleocytosis in two and oligoclonal bands in the last case. Follow up MRI was available in two patients, both reporting cerebellar atrophy. The 65-year-old woman had no improvement with steroids, while the other two patients were reported to have a partial improvement with immunotherapy which included a combination of steroids, IVIg, and mycophenolate mofetil (MMF) (17–19, 37).

Other Antibodies Associated With Cerebellar Ataxia

There are a handful of other autoantibodies that are associated with symptoms of cerebellar ataxia, although clinical manifestation of these antibody syndromes predominately present with extracerebellar phenotypes (**Table 2**). IgLON5 has a unique presentation consisting mainly of sleep disorders, bulbar symptoms and gait abnormalities (not necessarily all attributed to cerebellar dysfunction). Cerebellar ataxia in anti-IgLON5 disease is rare, however it has been described along with MRI findings of cerebellar atrophy (20). In anti-contactin-associated protein-like 2(Caspr2) antibody syndrome, unique symptoms such as peripheral nerve hyperexcitability and neuromyotonia tend to accompany the encephalitis and ataxia (8, 46). In a cohort of 37 patients with anti-Caspr2 antibodies (20 with encephalitis and 17 with neuromyotonia), 5 patients with transient symptoms suggestive of cerebellar impairment suggesting that cerebellar ataxia could be observed in up to 25% of patients (9). Kelch-like protein 11 (KLHL11) have been identified as a biomarker of a paraneoplastic brainstem cerebellar syndrome associated with testicular seminoma (23). While often presenting with cerebellar ataxia, more recently expanded phenotypes in KLHL11 have been described including co-existing anti-NMDAR encephalitis, brainstem diencephalic encephalitis, opsoclous-myoclonus, limbic and extralimbic encephalitis and chronic psychosis in the setting of teratomas (24). Autoimmune glial fibrillary acidic protein (GFAP) astrocytopathy is a newly described autoimmune meningoencephalomyelitis syndrome associated with ataxia in 40% of reported cases (13).

In terms of paraclinical data, the majority of patients with GFAP and KLHL11 antibodies had an inflammatory CSF profile (including pleocytosis, elevated protein, OCBs and/or elevated IgG index), while only about half of patients with IgLON5 and a quarter of patients with Caspr2 antibodies had these same abnormalities. Cerebellar atrophy was one of the more common MRI findings along with T2/FLAIR signal changes in the cerebellum, brainstem, or hippocampi. In GFAP astrocytopathy the majority of patients have characteristic T1 postgadolinium enhancement including patterns of radial periventricular, leptomeningeal and punctate, and serpiginous and periependymal enhancement; spinal cord enhancement can also be seen often with a central cord pattern (13).

DISCUSSION

Here we have reviewed recently described autoantibodies associated with cerebellar ataxia including anti-AP3B2, anti-ITPR1, anti-TRIM, anti-neurochondrin, anti-NIF, anti-septin 5,anti-mGluR2, anti-SEZ6L2, and anti-homer 3. Clinically, patients present with a subacute to rapidly progressive cerebellar ataxia. The majority of cases reported an inflammatory CSF

profile with a lymphocytic pleocytosis and/or the presence of unique oligoclonal bands. Imaging abnormalities are more variable but when present often involve T2-hyperintensities or atrophy of the cerebellum.

Main differences between the antibodies described lies in their associations with malignancy and reported responses to treatment. Malignancy was less common in patients with anti-AP3B2 antibody-associated cerebellar ataxia with only 1 in 9 patients identified as having an underlying cancer. On the other hand, anti-NIF antibodies are strongly associated with neuroendocrine tumors and the few patients described with anti-TRIM antibodies were all found to have lung adenocarcinomas. ITPR1 antibodies seem to be associated with breast cancer although reports of malignancy types were more variable. Both patients with mGluR2 antibodies had malignancies of different types. No malignancies have been reported with anti-neurochondrin and with anti-SEZ6L2 only 1 patient was identified with a ovarian cancer, diagnosed 4 years later. In contrast, KLHL11 has a strong association with testicular seminoma in men.

Most of the described antibodies in this review, except for mGluR2 and SEZ6L2 (see details in **Table 2**), are directed against intracellular antigens. It is widely hypothesized, that antibodies against intracellular targets are not directly pathogenic and rather mediate damage via cytotoxic T-cells, although there has been some evidence to dispute this assumption. For example, studies have shown that neurons are able to take up IgG containing anti-Yo, which subsequently resulted in Purkinje cell death (39). The same direct cytotoxic effect has also been found with anti-Hu antibodies (27), although it remains unclear exactly how these antibodies cause destruction of neurons. Regardless of whether the antibodies themselves or cytotoxic T-cells are mediating the damage, these conditions are often reported to be less responsive to immunotherapies. This appears to hold true of the newly discovered antibodies described in this review with the only exception being anti-NIF antibody cerebellar ataxias, where most patients had improvement of symptoms with immunotherapy. Furthermore, while SEZ6L2 is an extracellular antibody, the immunotherapy response in the 4 reported patients was poor.

In general, autoimmune and paraneoplastic cerebellar ataxias result in progressive and debilitating symptoms, therefore, it is crucial for clinicians to be aware of this diagnosis so treatment can be started as early as possible, ideally before significant, irreversible neuronal cell death has taken place. Any patient presenting with a subacute onset of cerebellar ataxia without a family history of genetic ataxia, an autoimmune pathogenesis should be considered. The absence of an antibody does not exclude neurological autoimmunity and if suspected immunotherapy should be considered, especially if the diagnostic work up reveals an inflammatory CSF profile. Equally important, these patients need screening for underlying malignancy as the neurologic syndrome is often the first indication of a neoplasm. There remains much to learn regarding pathogenesis and exact mechanisms of neuronal destruction so more effective therapies can be investigated, ideally through robust randomized-controlled trials.

AUTHOR CONTRIBUTIONS

MG and AP drafted and revised the manuscript for intellectual content and direct patient care. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported in part by the Drake family in the name of Susan Drake and through the Rocky Mountain MS Center at the University of Colorado.

ACKNOWLEDGMENTS

We would like to thank Dr. Christopher Bartley and colleagues in Dr. Michael Wilson's laboratory at the University of San Francisco (USCF) Weill Institute for Neurosciences for providing novel autoantibody testing and the images provided in **Figure 3**. The authors would also like to thank Dr. Divyanshu Dubey and colleagues at the Mayo Clinic Neuroimmunology Laboratory for providing identification of the unclassified antibodies in both clinical cases presented in this manuscript.

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frontiers in Neurology

ORIGINAL RESEARCH
published: 26 August 2021
doi: 10.3389/fneur.2021.731835

Analysis of Predictive Risk Factors in Aquaporin-4-IgG Positive Highly Active Neuromyelitis Optica Spectrum Disorders

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OPEN ACCESS

Edited by:
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Reviewed by:
Wakiro Sato, National Center of Neurology and Psychiatry, Japan
Mitsuru Watanabe, Kyushu University, Japan

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

Received: *28 June 2021*
Accepted: *04 August 2021*
Published: *26 August 2021*

Citation:
Li Y, Zhang J, Zhou Y, Xie H, Duan R, Jing L, Yao Y, Teng J and Jia Y (2021) Analysis of Predictive Risk Factors in Aquaporin-4-IgG Positive Highly Active Neuromyelitis Optica Spectrum Disorders. Front. Neurol. 12:731835. doi: 10.3389/fneur.2021.731835

Neuromyelitis optica spectrum disorders (NMOSDs) are inflammatory diseases with a high risk of recurrence and progressive disability, and it is crucial to find sensitive and reliable biomarkers for prognosis and the early prediction of relapse. Highly active NMOSD is defined as two or more clinical relapses within a 12-month period. In this study, we analyzed independent risk factors among patients with aquaporin-4 (AQP4)-IgG positive highly active NMOSD. In this retrospective study, we analyzed the data of 94 AQP4-IgG positive patients with highly active NMOSD and 105 AQP4-IgG positive controls with non-highly active NMOSD. In order to rule out possible effects of previous treatments (such as glucocorticoids, immunoglobulin, and immunosuppressants), we focused on the first-attack NMOSD patients admitted to our hospital. Clinical data, including the age of onset, gender, comorbidities, and serum analysis and cerebrospinal fluid (CSF) analysis results, were collected, after which logistic regression models were used to determine the associations between the clinical factors and relapse outcomes. The prevalence of connective tissue disease and the proportion of antinuclear antibody (ANA)-positivity were higher in the highly active NMOSD group than in the control group. The leukocyte counts, homocysteine (Hcy) levels, CSF leukocyte counts, protein concentrations, IgG indexes, and 24h IgG synthesis rates were also higher in the highly active NMOSD group. The results of multivariate analysis indicated that connective tissue disease comorbidity (OR = 5.953, 95% CI: 1.221–29.034, $P = 0.027$), Hcy levels (OR = 1.063, 95% CI: 1.003–1.126, $P = 0.04$), and 24h IgG synthesis rate (OR = 1.038, 95% CI: 1.003–1.075, $P = 0.034$) may be independent risk factors for AQP4-IgG positive highly active NMOSD relapse after adjusting for various variables. Comorbidity of connective tissue disease, Hcy levels, and 24h IgG synthesis rate may be independent risk factors for AQP4-IgG positive highly active NMOSD.

Keywords: neuromyelitis optica spectrum disorders, aquaporin-4, relapse, risk factors, comorbidities, homocysteine levels, 24h IgG synthesis rates

INTRODUCTION

Neuromyelitis optica spectrum disorders (NMOSD) are autoimmune diseases characterized by unpredictable attacks on the optic nerves, spinal cord, brainstem, and other areas of the central nervous system, which result in an accumulation of neurological disability. NMOSD has a wide spectrum of clinical features, including optic neuritis, longitudinally extensive transverse myelitis, diencephalic syndrome, and other encephalitic presentations. Patients with NMOSD have a high risk of recurrence and a high incidence of disability. The prognosis of relapsing NMOSD is usually poor, particularly among patients with frequent relapses (1–3). Therefore, researchers have attempted to find reliable and sensitive markers to predict NMOSD relapses and prognosis.

Highly active NMOSD, also known as highly relapsing NMOSD, is defined as at least two clinical relapses during the previous 12 months. Previous studies showed that the comorbidity burden was significantly higher among patients with highly active NMOSD compared with the overall NMOSD population (4, 5). However, the association between comorbidity and relapse has not been further analyzed.

Several predictive factors for the prognosis of NMOSD have previously been identified. For example, antinuclear antibodies (ANAs) were found to be related to more severe disease activity in NMOSD patients (6). Disease duration of NMOSD was shorter in ANA (+) patients with an Expanded Disability Status Scale (EDSS) value < 4 than in ANA (–) patients (7). Serum homocysteine (Hcy) levels were significantly higher in patients with NMOSD with an EDSS value ≥ 4 in the acute stage, indicating that Hcy may play an important role in the progression of NMOSD (8). However, no prior study has comprehensively investigated the risk factors affecting patients with highly active NMOSD in a real-world setting.

In this study, we conducted a retrospective analysis to explore predictive risk factors among patients with aquaporin-4 (AQP4)-IgG positive highly active NMOSD. We focused on the first-attack NMOSD patients admitted to our hospital in order to rule out possible effects of previous treatments (such as glucocorticoids, immunoglobulin, and immunosuppressants) and accurately calculate the times of relapse events during the follow-up.

METHODS

Participants

Patients diagnosed with NMOSD at the First Affiliated Hospital of Zhengzhou University between January 2013 and December 2019 were enrolled in this study. NMOSD was diagnosed based on the 2015 International Consensus Diagnostic Criteria for NMOSD (9). We screened patients who had experienced their first attack and divided them into two groups according to the number of relapses: patients who experienced two or more clinical relapses within 12 months following the first attack during the follow-up period were assigned to the highly active NMOSD group, while patients with no or one relapse within 12 months after the first attack were assigned to the control group (non-highly active NMOSD group). The exclusion criteria for both groups were as follows: (1) patients who had previously experienced NMOSD; (2) the coexistence of other diseases that may affect EDSS values; (3) taking corticosteroids or undergoing immunosuppressive therapy during the 6 months before admission; (4). using drugs that may affect laboratory tests such as lipid-lowering drugs, Hcy-lowering drugs, and hepatic and renal protectants before admission; (5) hematological, infectious, or other diseases that may affect blood test results and cerebrospinal fluid (CSF) analysis; (6) incomplete data at admission; (7) missing follow-up data; (8) patients who were AQP4-IgG negative or who were not tested. The detailed selection process is shown in **Figure 1**. The study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2019-KY-018) and was performed in accordance with the Declaration of Helsinki.

Data Collection

The patients' clinical data, including age of onset, gender, comorbidities, clinical symptoms, treatment, and laboratory test results (routine blood test, blood lipid, liver function, renal function, and thyroid hormone test, ANAs) and CSF analysis results (cells count, protein levels, IgG index and 24h synthesis rate) were collected. Blood samples were collected from patients after overnight fasting at 7:00–8:00 a.m. the next day after admission. Both blood and CSF samples were taken prior to treatment. Intrathecal immunoglobulin synthesis was detected using isoelectric focusing as previously described (10). The IgG index was calculated as the quotient of CSF-plasma concentration IgG divided by the CSF-plasma concentration quotient for albumin (QIgG/Qalb) (11). Anti-AQP4 antibodies in the serum or CSF were detected by an assay on live cells transfected with AQP4 at the Neurology Laboratory of the First Affiliated Hospital of Zhengzhou University (12). Other tests were performed in accordance with the manufacturer's protocols, and the examiners were blinded to the diagnoses and clinical symptoms.

Patients received different treatments according to their clinical symptoms and financial situation. Intravenous methylprednisolone or plasma exchange were used in most patients in the acute phase. Low dose oral prednisolone, azathioprine (2–3 mg/kg daily), mycophenolate mofetil (1–2 g daily), methotrexate (17.5 mg weekly) and rituximab were also used in part of patients to prevent attacks.

Outcomes

The primary outcome of this study was highly active relapse events. A relapse event was defined as new-onset or recurrent neurological symptoms that lasted for at least 24 h and caused an EDSS increase of at least 0.5 points from the lowest score. Relapse events occurring within 28 days were regarded as part of a single relapse (13). Each patient was diagnosed and had their EDSS scores evaluated by an experienced neurologist. Clinical

Abbreviations: NMOSD, neuromyelitis optica spectrum disorder; AQP-4, Aquaporin-4; ANA, Antinuclear antibody; EDSS, Expanded Disability Status Scale; Hcy, Homocysteine; CI, Confidence interval; OR, Odds ratios; CSF, Cerebrospinal fluid; SD, Standard deviation; IQR, Interquartile range; OB, Oligoclonal band; NMDAR, N-methyl-D-aspartate receptor; BBB, Blood brain barrier.

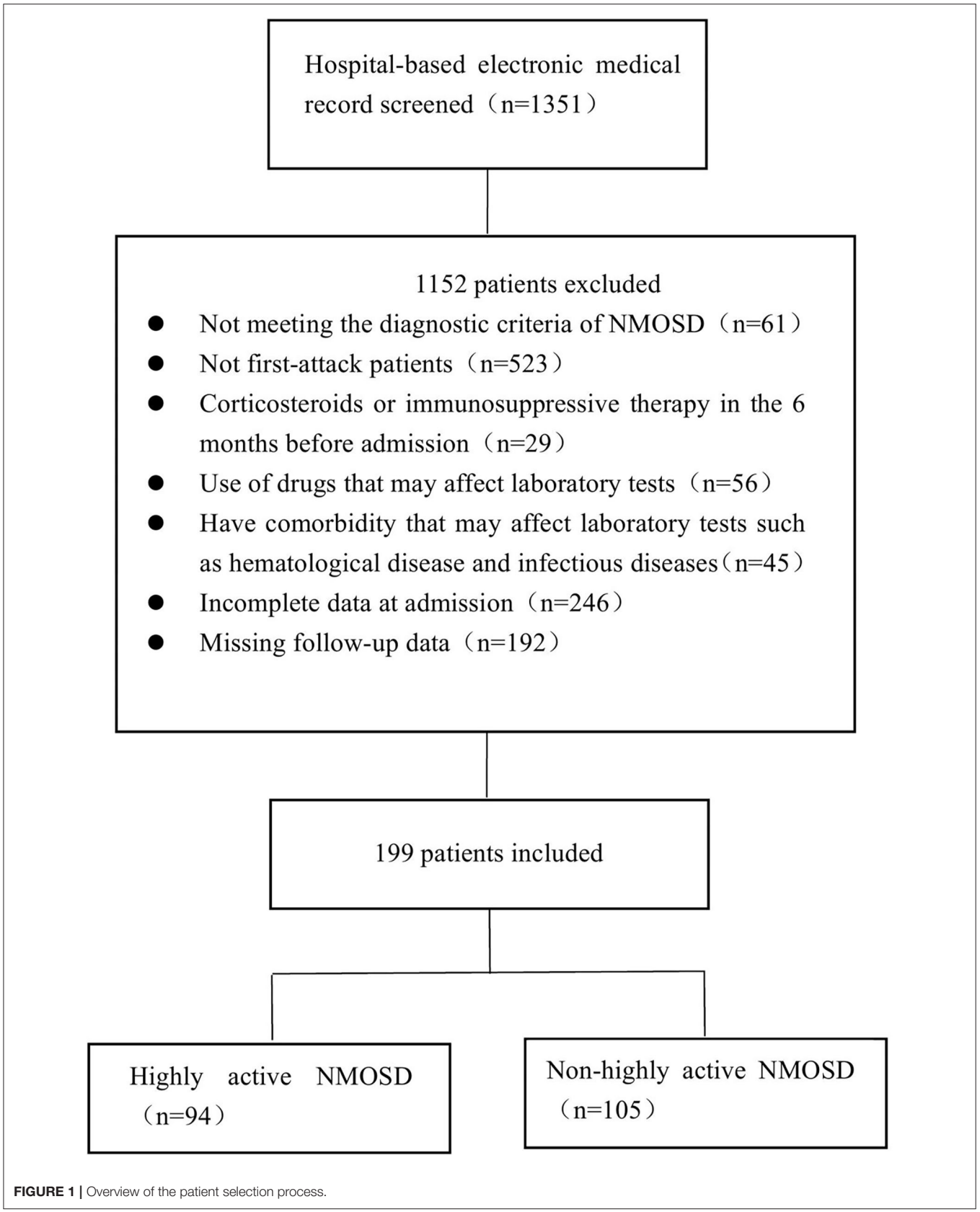

FIGURE 1 | Overview of the patient selection process.

symptoms at the time of patients' first attack before treatment were recorded as initial EDSS scores. Clinical symptoms at the final follow-up were recorded as final EDSS scores. Follow-up data were obtained through an annual clinic visit or a telephone interview every 3 months. The total follow-up duration was 12 months after first admission.

Statistical Analysis

Data were presented as mean ± standard deviation (SD) if the continuous variables were distributed normally according to the Kolmogorov–Smirnov test. Otherwise, data were presented as median [interquartile range (IQR)]. Classification variables were expressed as frequency (percentage, %). The differences between the two groups were analyzed using the Student's *t*-test and Wilcoxon test for normally and abnormally distributed data, respectively. Categorical data were compared using the chi-square test when comparing numbers ≥5 or Fisher's exact test when comparing small numbers < 5. Univariate logistic regression analysis was used to screen factors that might affect highly active NMOSD outcomes. Multivariate logistic regression was used to analyze the independent effects of variables on highly active relapse outcomes. Variables with $P < 0.2$ according to univariate logistic regression analysis or variables considered to be associated with relapse outcomes in NMOSD in previous studies, such as age, sex, hypertension, and initial EDSS, were included in the multivariate model. Receiver operating characteristic (ROC) curve analysis was performed to test the predictive ability of Hcy and the 24h IgG synthesis rate for highly active NMOSD and predict the optimal cut-off value of Hcy and the 24h IgG synthesis rate in patients with highly active NMOSD. $P < 0.05$ was considered significant. All analyses were performed using IBM SPSS Statistics 21 software and the diagram was generated with GraphPad Prism 8.

RESULTS

Demographic and Clinical Characteristics

In the database of the First Affiliated Hospital of Zhengzhou University from January 2013 to December 2019, 1,351 patients met the NMOSD diagnostic criteria. Of the 199 cases of AQP4-IgG positive first-attack NMOSD who were admitted to our hospital, 94 met the inclusion criteria for highly active NMOSD in accordance with the specified relapse times during the 12 month-follow-up after the first attack, and 105 AQP4-IgG positive cases were enrolled as controls (non-highly active NMOSD). Patients with highly active NMOSD experienced an average of 2.3 relapses during the 12-month follow-up period. As shown in **Table 1**, there were no significant differences in age, gender, and the number of patients who smoke and consume alcohol between the two groups ($P > 0.05$). The prevalence of hypertension, diabetes, coronary heart disease, cerebrovascular disease, and malignancy was not significantly different between the two groups ($P > 0.05$). The prevalence of connective tissue disease was significantly higher among the patients with highly active NMOSD than non-highly active NMOSD (15.96% for patients with highly active NMOSD, 2.86% for controls, $P = 0.002$). In highly acive NMOSD group, 15 cases presented with connective tissue disease (three cases with systemic lupus erythematosus, three cases with rheumatoid arthritis, and nine cases with Sjögren syndrome). In the non-highly acive NMOSD group, three cases presented with connective tissue disease (one cases with systemic lupus erythematosus, two cases with Sjögren syndrome) (**Figure 2**).

Clinical symptoms of all patients were evaluated at the time of their first attack before treatment and recorded as an initial EDSS score, which did not differ significantly between the groups. Patients received different treatments according to their clinical symptoms and financial situation, such as corticosteroids, immunoglobulin, and immunosuppressants (azathioprine, mycophenolate mofetil, methotrexate, and rituximab). There were no statistically significant differences in these parameters between the two groups. At the final follow-up, the final EDSS score of the highly active NMOSD group was significantly higher than that of the controls ($P < 0.001$; **Table 1**).

Regarding the laboratory test results of the study groups (**Table 2**), there were no significant differences in the erythrocyte, hemoglobin, lymphocyte, and glycosylated hemoglobin, folic acid, and vitamin B12 of the two groups. The proportion of patients with hepatic dysfunction, renal dysfunction, high triglyceride and total cholesterol levels, and positive thyroid peroxidase or thyroglobulin antibodies was similar between the two groups. The proportion of ANA-positive patients was higher in the highly active NMOSD group than in the control group. Leukocyte counts, Hcy levels, CSF leukocyte counts and protein concentration, IgG index, and 24h IgG synthesis rate were higher in the highly active NMOSD group than in the control group.

Predictors Associated With the Occurrence of Highly Active NMOSD

To explore potential risk factors that may predict the occurrence of highly active NMOSD, univariate logistic regression analysis was performed. Analyses showed that connective tissue disease [OR (odds ratios) = 6.456, 95% CI (Confidence interval): 1.806–23.078, $P = 0.004$], serum leukocyte counts (OR = 1.119, 95% CI: 1.012–1.238, $P = 0.028$), serum Hcy levels (OR = 1.095, 95% CI: 1.039–1.154, $P = 0.001$), ANAs (OR = 2.355, 95% CI: 1.14–4.864, $P = 0.021$), CSF protein concentration (OR = 1.001, 95% CI: 1.000–1.003, $P = 0.016$), 24h IgG synthesis rate (OR = 1.058, 95%CI: 1.022–1.096, $P = 0.002$) were significantly correlated to the occurrence of highly active NMOSD (**Table 3**).

Variables with a significance of $P < 0.2$ according to univariate logistic regression analysis or variables considered to be associated with relapses in NMOSD in previous studies, such as age, gender, hypertension, and initial EDSS, were included in the multivariate model. Multivariate logistic regression analysis showed that connective tissue disease (OR = 5.953, 95% CI: 1.221–29.034, $P = 0.027$), Hcy levels (OR = 1.063, 95% CI: 1.003–1.126, $P = 0.04$), and 24h IgG synthesis rate (OR = 1.038, 95% CI: 1.003–1.075, $P = 0.034$) were significantly correlated with the occurrence of highly active NMOSD (**Table 4**; **Figure 3**).

TABLE 1 | Comparison of clinical data between patients with highly active NMOSD and non-highly active NMOSD patients.

	Total patients ($n = 199$)	Highly active NMOSD ($n = 94$)	Non-highly active NMOSD ($n = 105$)	*P*-value
Age in years (mean ± SD)	41.02 ± 15.49	39.88 ± 16.85	42.04 ± 14.17	0.333
Gender, female, *n* (%)	171 (85.93)	81 (86.17)	90 (85.71)	0.926
Smoking, *n* (%)	12 (6.03)	4 (4.26)	8 (7.62)	0.382
Alcohol consumption, *n* (%)	5 (2.51)	2 (2.13)	3 (2.86)	1.000
Comorbidities				
Hypertension, *n* (%)	21 (10.55)	8 (8.51)	13 (12.38)	0.375
Diabetes, *n* (%)	13 (6.53)	7 (7.47)	6 (5.71)	0.621
Coronary heart disease, *n* (%)	4 (2.01)	1 (1.06)	3 (2.86)	0.624
Cerebrovascular disease, *n* (%)	5 (2.51)	3 (3.19)	2 (1.90)	0.669
Malignancy, *n* (%)	4 (2.01)	2 (2.13)	2 (1.90)	1.000
[a]Connective tissue disease, *n* (%)	17 (8.54)	15 (15.96)	3 (2.86)	0.002*
Onset attack, *n* (%)				
Optic neuritis	72 (36.18)	40 (42.55)	32 (30.48)	0.077
Transverse myelitis	148 (74.37)	70 (74.47)	78 (74.29)	0.977
Initial EDSS	5.16 ± 1.84	5.33 ± 1.75	5.02 ± 1.91	0.235
Treatment, *n* (%)				
Corticosteroid	186 (93.47)	88 (93.62)	98 (93.33)	0.936
Intravenous immunoglobulin	16 (8.04)	9 (9.57)	7 (6.67)	0.463
Immunosuppressant	66 (33.17)	31 (32.98)	35 (33.33)	0.92
Azathioprine	41 (20.60)	19 (20.21)	22 (20.95)	0.898
Mycophenolate mofetil	21 (10.55)	10 (10.64)	11 (10.48)	0.97
Rituximab	2 (1.01)	1 (1.06)	1 (0.95)	1.000
Methotrexate	2 (1.01)	1 (1.06)	1 (0.95)	1.000
Final EDSS	4.633 ± 1.93	5.42 ± 1.66	3.92 ± 1.89	<0.001*

[a]*Connective tissue disease consists of systemic lupus erythematosus, rheumatoid arthritis, and Sjögren syndrome.*
*$P < 0.05$.

ROC Curve for Hcy and 24h IgG Synthesis Rate at Admission Predicts the Occurrence of Highly Active NMOSD

ROC curve analysis was used to evaluate the predictive value of Hcy and 24h IgG synthesis rate in patients with highly active NMOSD. The area under the ROC curve was 0.654 (95% CI: 0.577–0.731, $P < 0.001$) and 0.749 (95% CI: 0.679–0.819, $P < 0.001$) for Hcy levels and 24h IgG synthesis rates, respectively. At an Hcy cut-off value of 14.735 μmol/L, the sensitivity for predicting the occurrence of highly active NMOSD was 47.9%, and the specificity was 82.9%. At a 24h IgG synthesis rate cut-off value of 2.815, the sensitivity for predicting highly active NMOSD was 77.7%, and the specificity was 69.5% (**Figure 4**).

DISCUSSION

NMOSDs are severe inflammatory disorders of the central nervous system that mainly affect the optic nerves and spinal cord, which causes severe and irreversible disabilities (14, 15), and limited reliable prognostic indicators currently exist. In this study, we performed a retrospective analysis to explore independent risk factors among patients with highly active NMOSD compared to those with non-highly active NMOSD. We analyzed patients' clinical characteristics, laboratory test results, and relapse information and found that the prevalence of connective tissue disease, leukocyte counts, Hcy levels, the proportion of ANA-positive patients, CSF leukocyte counts, protein concentration, IgG index, and 24h IgG synthesis rate were higher in the highly active NMOSD group than among the non-highly active NMOSD patients. Logistics regression analysis indicated that the prevalence of connective tissue disease, Hcy levels, and 24h IgG synthesis rate might be independent risk factors for highly frequent relapse events in patients with first-attack NMOSD during the 12 month follow-up period. The optimal cut-off values of Hcy and the 24h IgG synthesis rate for predicting highly active NMOSD were 14.735 μmol/L and 2.815 mg/24h, respectively. To the best of our knowledge, this is the first study to investigate the relationship between the prevalence of comorbidities, laboratory results, and relapse events in NMOSD and to determine whether these indicators are risk factors among patients with highly active NMOSD in a real-world setting. In order to eliminate the effects of previous treatments (such as glucocorticoids, immunoglobulin, and immunosuppressants) and accurately calculate the times of relapse events during the follow-up period, we focused on patients with first-attack NMOSD in the study.

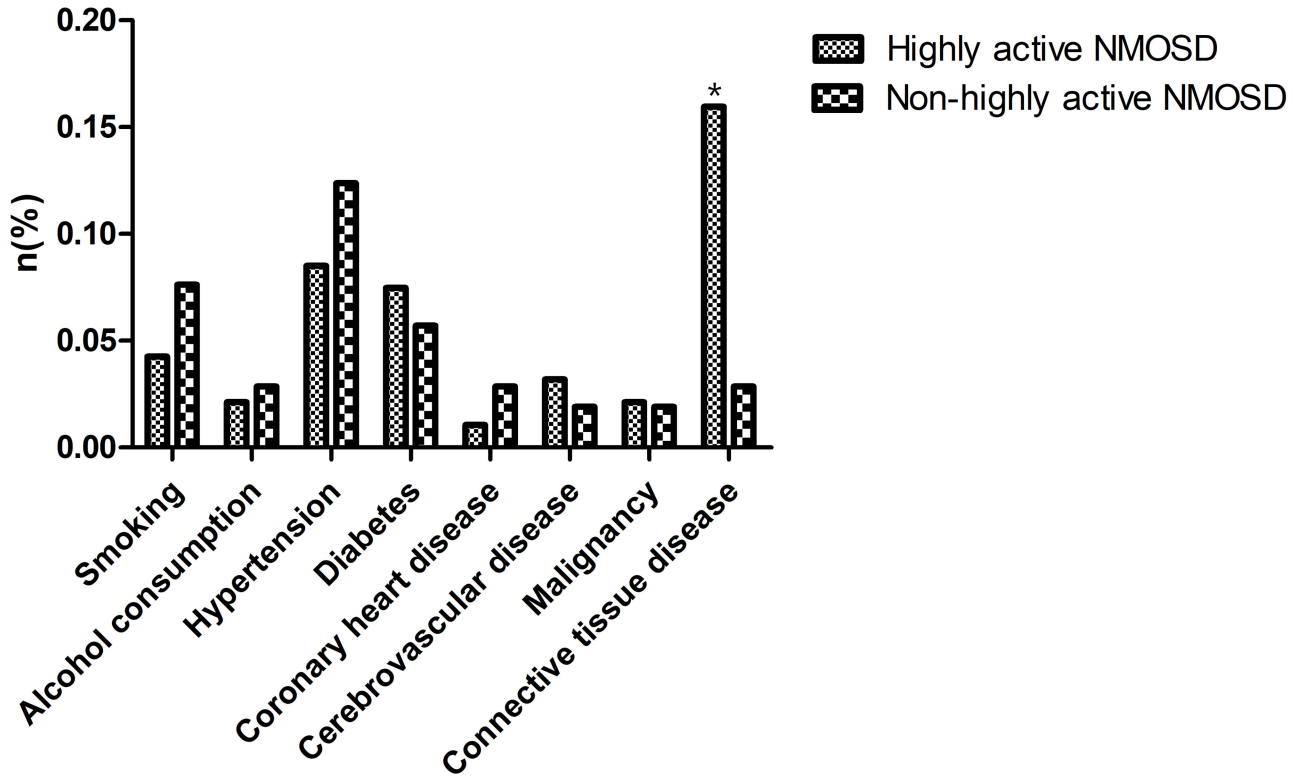

FIGURE 2 | Comorbidities of the patients. The prevalence of connective tissue disease was significantly higher among the patients with highly active NMOSD than non-highly active NMOSD. *$P < 0.05$.

In the database of the First Affiliated Hospital of Zhengzhou University from January 2013 to December 2019, 1,351 patients were preliminarily enrolled, and 1,290 patients met the NMOSD diagnostic criteria, therefore, we collected their data. After our strict screening process, 199 cases of first-attack NMOSD were included in the study. Prior evidence suggests that the female-to-male ratio among NMOSD patients is about 8:1 for AQP4-IgG seropositive patients and 2:1 for AQP4-IgG seronegative patients (16). Our findings showed that, among cases of highly active NOMSD, the proportion of females was higher (6.23:1) compared to that of the previous report (3.95:1) (4). However, the logistic regression analysis results indicated that sex did not significantly affect highly active NMOSD (OR = 1.095, 95% CI: 0.403–2.974, $P = 0.859$). We speculate that different survey regions may account for the difference in the female-to-male ratio. In this study, we mainly focused on the population of the central region of China, taking the geographical location of our hospital into account. The mean age of patients with highly active NMOSD was 39.88, similar to that of previous studies.

Previous studies have demonstrated that comorbidities such as vascular disease, may potentially explain the heterogeneity in multiple sclerosis (MS) outcomes (17). It was also reported that the comorbidity burden was greater among patients with highly active NMOSD than among the overall NMOSD population (4). Thus, we investigated whether comorbidities are associated with relapse and progression in NMOSD. Previous studies have shown that the most common comorbidities that coexist with NMOSD are autoimmune diseases, such as systemic lupus erythematosus, Sjögren syndrome, rheumatoid arthritis, and others (18–21). Our results were consistent with those of previous reports in that we found that the prevalence of connective tissue diseases was greater among patients with highly active NMOSD than in patients with non-highly active NMOSD. Furthermore, the multivariate analysis results showed that connective tissue diseases were independent risk factors for highly active NMOSD, suggesting that connective tissue diseases may be associated with a high risk of relapse in NMOSD. One possible mechanism is the activation of autoreactive Th1 cells and B cells via Th2 cells in autoimmune diseases (22). On the other hand, it is believed that ANAs are the most common biomarkers of these connective tissue diseases, which could cause inflammation and tissue damage by cross-reactivity and forming immune complexes of antibodies with DNA or nucleosomes. The proportion of ANA-positive patients is between 27.3 and 82.6% (23–25), whereas in our study, the proportion was 19.6%, which is lower than that of previous reports. Since ANA tests were not included in the conventional examination, many patients without ANA results were excluded during the screening process, which may account for the lower proportion of ANA-positive patients in our study. ANAs are reportedly related to more severe disease activity in patients with NMOSD according to a previous study (7). In

TABLE 2 | Comparison of laboratory tests between patients with highly active NMOSD and non-highly active NMOSD patients.

	Total patients ($n = 199$)	Highly active NMOSD ($n = 94$)	Non-highly active NMOSD ($n = 105$)	*P*-value
Leukocyte counts, median (IQR) ($\times 10^9$/L)	6.4 (5.0–7.9)	6.7 (5.5–8.9)	6.0 (4.7–7.5)	0.036*
Erythrocyte counts, median (IQR) ($\times 10^{12}$/L)	4.18 (3.91–4.52)	4.15 (3.93–4.97)	4.18 (3.90–4.56)	0.319
Hemoglobin, median (IQR) (g/L)	125 (116–135)	126 (116–134.5)	125 (116–135)	0.787
Lymphocyte counts, median (IQR) ($\times 10^9$/L)	1.79 (1.23–2.48)	1.82 (1.315–2.67)	1.72 (1.16–2.35)	0.385
Glycosylated hemoglobin, median (IQR)	5.70 (5.40–6.08)	5.77 (5.36–6.13)	5.67 (5.4–5.91)	0.209
[a]Hepatic dysfunction, *n* (%)	17 (8.54)	10 (10.64)	7 (6.67)	0.317
[b] Renal dysfunction, *n* (%)	4 (2.01)	2 (2.13)	2 (1.90)	1.000
High triglyceride, *n* (%)	31 (15.58)	13 (13.83)	18 (17.14)	0.520
High total cholesterol, *n* (%)	41 (20.60)	17 (18.09)	24 (22.86)	0.406
Homocysteine levels, median (IQR) (μmol/L)	11.95 (9.27–16.26)	14.03 (9.89–18.83)	10.87 (8.94–13.32)	<0.001*
Folic acid, median (IQR) (ng/mL)	7.3 (4.70–11.23)	6.5 (4.48–10.45)	7.78 (5.08–11.45)	0.208
Vitamin B12, median (IQR) (pg/mL)	516.2 (346.0–788.7)	555.65 (337.25–836.85)	467 (356–728.2)	0.222
ANA-positive, *n* (%)	39 (19.60)	25 (26.60)	14 (13.33)	0.019*
Thyroid peroxidase / thyroglobulin antibodies positive, *n* (%)	17 (8.84)	8 (8.51)	9 (8.57)	0.988
Cerebrospinal fluid leukocyte counts, median (IQR) ($\times 10^6$/L)	6 (2–19.25)	12 (6–25)	4 (2–11)	<0.001*
Cerebrospinal fluid protein concentration, median (IQR) (mg/L)	396.4 (284–582.6)	460.85 (328.45–653.25)	346.1 (235.55–503.5)	<0.001*
IgG index, median (IQR)	0.63 (0.53–0.78)	0.715 (0.58–0.84)	0.58 (0.51–0.71)	<0.001*
24h IgG synthesis, median (IQR) (mg/24h)	3.12 (0–10.23)	7.74 (2.97–15.22)	1.37 (0–3.87)	<0.001*

[a]*Hepatic dysfunction refers to abnormal transaminase and bilirubin level.*
[b]*Renal dysfunction refers to abnormal creatinine and urea nitrogen level.*
*$P < 0.05$.

our study, univariate analysis also revealed that ANAs were risk factors in patients with highly active NMOSD (OR = 2.355, 95% CI: 1.1–4.864, $P = 0.021$), but no significant correlation was found during multivariate analysis.

The role of serum Hcy in autoimmune demyelinating diseases of the central nervous system has recently attracted more attention. An updated meta-analysis indicated that elevated Hcy levels might affect the pathogenesis or progression of MS. The prognosis of MS patients with hyperhomocysteinemia was worse than that of MS patients with lower Hcy levels in terms of disease progression (26). Hcy levels are associated with the progression of NMOSD. A previous study reported that serum Hcy levels were higher in patients with acute-phase NMOSD who had severe initial symptoms (EDSS score ≥ 4) than in patients with mild initial symptoms (EDSS score < 4), and EDSS was positively correlated with Hcy levels in acute-phase NMOSD (7). In our study, we found that serum Hcy levels were higher in the patients with highly active NMOSD than in patients with non-highly active NMOSD. Moreover, Hcy level was an independent risk factor among patients with highly active NMOSD, suggesting that those with higher Hcy levels may have a higher relapse rate during the first 12 months following the first attack. This is the first study to investigate Hcy levels in patients with highly active NMOSD and the relationship between Hcy levels and relapse events.

The effect of Hcy in highly active NMOSD is still not clear. Possible underlying mechanisms of higher Hcy leading to relapse are presented as follows. Elevated Hcy levels can cause oxidative stress, and mitochondrial dysfunction by increasing ROS production (27, 28). Hcy is an agonist of glutamate receptors [N-methyl-D-aspartate receptor (NMDAR)], the stimulation of which can cause excitotoxicity, the activation of caspases and increase of the intracellular calcium concentration, inducing neuronal injury and apoptosis. Hcy can exert pro-inflammatory effects by modulation of adaptive immune system cell function and disturbing the blood brain barrier (BBB). Th17-cells have been identified to be able to destroy BBB by producing pro-inflammatory IL-17 and migrate into CNS by expressing chemokine receptor CCR-6 (CD196). Hcy can affect the BBB functioning through activating the Th17-immune response. In addition (29). Hcy plays an important role in structural instability and degeneration of myelin sheath by inhibiting methyl donors, which may have an adverse effect on disease progression (30–32).

A double-blinded clinical trial demonstrated that administering vitamin B12 supplements and folate might reduce serum Hcy levels in MS patients and improve the physical and mental aspects of their quality of life (33, 34). Based on our results, we recommend that more positive treatment strategies should be considered for patients with NMOSD who have higher Hcy levels to reduce the occurrence of relapse events.

The normal range of intrathecal IgG synthesis is between −9 and 3.3 mg/24h (35). An elevated IgG synthesis rate has been reported in over 90% of patients with clinically definite MS.

TABLE 3 | Univariate logistic regression analysis of potential risk factors that may predict high relapse events in patients with NMOSD.

Variables	Univariate analysis	
	OR (95% CI)	*P*-value
Age	0.991 (0.973–1.009)	0.327
Gender, female	1.038 (0.466–2.314)	0.926
Smoking	0.539 (0.157–1.851)	0.326
Drinking	0.739 (0.121–4.522)	0.744
Hypertension	0.658 (0.260–1.666)	0.378
Diabetes	1.328 (0.430–4.101)	0.622
Coronary heart disease	0.366 (0.037–3.576)	0.387
Cerebrovascular disease	0.589 (0.096–3.604)	0.567
Malignancy	1.12 (0.155–8.109)	0.911
[a]Connective tissue disease	6.456 (1.806–23.078)	0.004*
Optic neuritis	1.69 (0.943–3.027)	0.078
Transverse myelitis	1.01 (0.534–1.91)	0.977
Initial EDSS	1.097 (0.942–1.278)	0.234
Corticosteroid	1.048 (0.339–3.236)	0.936
Intravenous immunoglobulin	1.467 (0.524–4.109)	0.466
Immunosuppressant	0.97 (0.537–1.753)	0.920
Leukocyte counts	1.119 (1.012–1.238)	0.028*
Erythrocyte counts	0.635 (0.374–1.077)	0.092
Hemoglobin	0.002 (0.974–1.009)	0.347
Lymphocyte counts	1.123 (0.826–1.528)	0.460
Glycosylated hemoglobin	1.161 (0.782–1.722)	0.460
Hepatic dysfunction	1.667 (0.608–4.571)	0.321
Renal dysfunction	1.12 (0.155–8.109)	0.911
High triglyceride	0.776 (0.357–1.684)	0.521
High total cholesterol	0.745 (0.372–1.493)	0.407
Homocysteine levels	1.095 (1.039–1.154)	0.001*
Folic acid	0.977 (0.917–1.040)	0.464
Vitamin B12	1.000 (1.000–1.001)	0.240
ANAs positive	2.355 (1.14–4.864)	0.021*
Thyroid peroxidase/thyroglobulin antibodies positive	0.992 (0.367–2.686)	0.988
Cerebrospinal fluid leukocyte counts	1.006 (0.997–1.015)	0.187
Cerebrospinal fluid protein concentration	1.001 (1.000–1.003)	0.016*
IgG index	1.555 (0.82–2.948)	0.176
24h IgG synthesis rate	1.058 (1.022–1.096)	0.002*

[a]*Connective tissue disease consists of systemic lupus erythematosus, rheumatoid arthritis, and Sjögren syndrome.*
*$P < 0.05$.

Considerable evidence indicates that IgG synthesis reflects the degree of chronic inflammation in the central nervous system and can be used as a monitoring marker for the progression of MS (35). Quantification of CSF oligoclonal bands (OBs) is a prognostic indicator in MS; patients with reduced or no OBs tend to have a better prognosis (36). Since the IgG daily synthesis rate was strongly correlated to OBs, we examined whether IgG synthesis predicts the prognosis of NMOSD. We found that the intrathecal IgG synthesis rate was higher in patients with highly active NMOSD than in patients with non-highly active NMOSD. We further demonstrated that a higher IgG synthesis rate may be related to a higher possibility of relapse in NMOSD.

This study has several limitations that should be noted. First, the total number of subjects included in the analyses was small, and the patients were from a single center. Also, we mainly focused on patients with highly active NMOSD who experienced two or more clinical relapses within 12 months following the first attack, and the follow-up period was relatively short. The results need to be further validated in larger, multicenter studies with longer follow-up periods. Second, quantitative analysis of OBs is not performed at our hospital, therefore, we failed to include this parameter in our study, which may be a source of bias in our results. Finally, we only included AQP4-IgG positive patients in the study, and the risk factors should also be studied in AQP4-IgG negative patients to analyze the results more accurately.

TABLE 4 | Multivariate logistic regression analysis of potential risk factors to predict high relapse events in patients with NMOSD.

Variables	Multivariate analysis			
	[a]Basic model		[b]Adjust I model	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Age			0.993 (0.971–1.017)	0.577
Gender, female			0.905 (0.39–2.416)	0.841
Hypertension			0.578 (0.18–1.856)	0.357
[c]Connective tissue disease	5.009 (1.095–22.915)	0.038*	5.953 (1.221–29.034)	0.027*
Optic neuritis			1.708 (0.875–3.336)	0.117
Initial EDSS			1.003 (0.825–1.219)	0.979
Leukocyte counts	1.086 (0.971–1.214)	0.147	1.12 (0.991–1.267)	0.07
Erythrocyte counts			0.582 (0.294–1.152)	0.12
Homocysteine levels	1.077 (1.020–1.137)	0.008*	1.063 (1.003–1.126)	0.04*
ANAs positive	1.149 (0.44–3.003)	0.777	0.853 (0.298–2.439)	0.766
Cerebrospinal fluid leukocyte counts			1.005 (0.995–1.015)	0.349
Cerebrospinal fluid protein concentration	1.001 (0.999–1.002)	0.468	1.001 (0.999–1.002)	0.419
IgG index			1.24 (0.651–2.362)	0.514
24h IgG synthesis rate	1.045 (1.010–1.082)	0.012*	1.038 (1.003–1.075)	0.034*

[a]Basic Model: Variables with P < 0.05 in the univariate logistic regression analysis were included in the multivariate model.
[b]Adjust I model: Variables with P < 0.2 in the univariate logistic regression analysis or variables considered to be associated with NMOSD relapse outcomes in previous studies, such as age, gender, hypertension, and initial EDSS, were included in the multivariate model.
[c]Connective tissue disease consists of systemic lupus erythematosus, rheumatoid arthritis, and Sjögren syndrome.
**P < 0.05.*

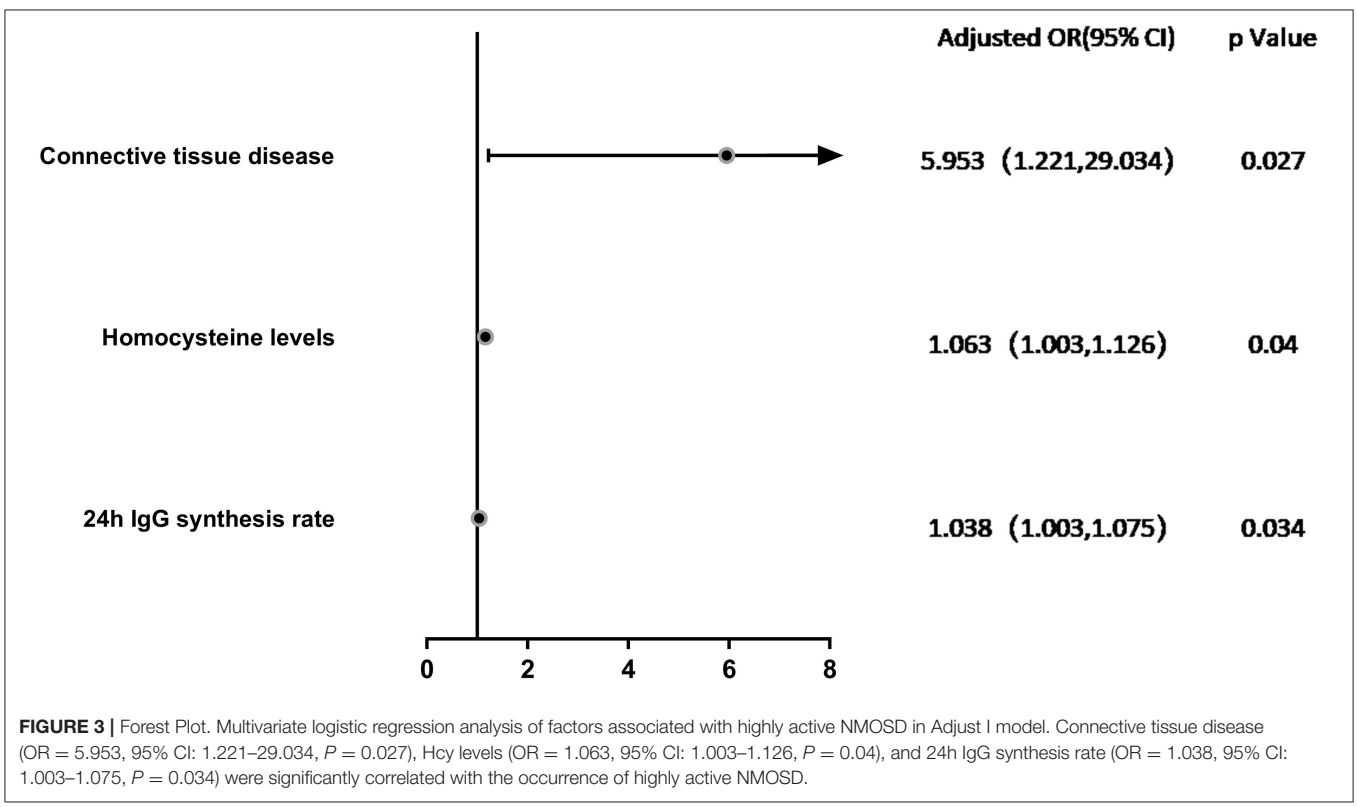

FIGURE 3 | Forest Plot. Multivariate logistic regression analysis of factors associated with highly active NMOSD in Adjust I model. Connective tissue disease (OR = 5.953, 95% CI: 1.221–29.034, *P* = 0.027), Hcy levels (OR = 1.063, 95% CI: 1.003–1.126, *P* = 0.04), and 24h IgG synthesis rate (OR = 1.038, 95% CI: 1.003–1.075, *P* = 0.034) were significantly correlated with the occurrence of highly active NMOSD.

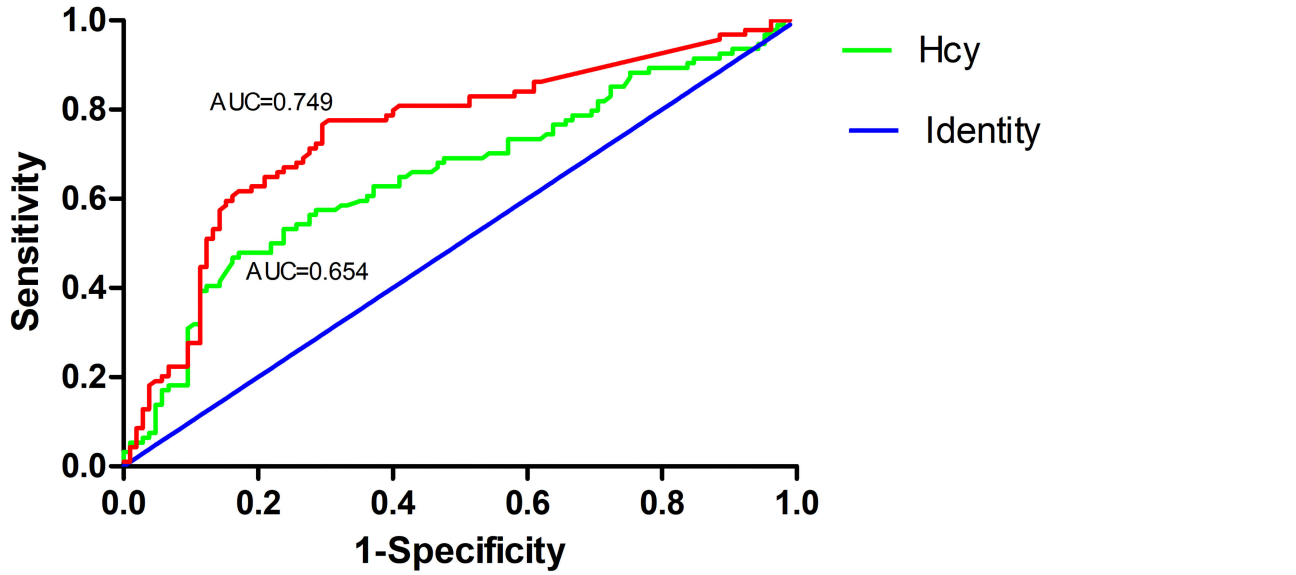

FIGURE 4 | Receiver operating characteristic (ROC) curve showing the predictive ability of Hcy and 24h IgG synthesis rate for highly active NMOSD. The area under the ROC curve was 0.654 and 0.749 for Hcy levels and 24h IgG synthesis rates, respectively.

In conclusion, we demonstrated that connective tissue disease comorbidity, Hcy levels, and 24h IgG synthesis rate may be independent risk factors in patients with AQP4-IgG positive highly active NMOSD. High Hcy levels and 24h IgG synthesis rates are associated with a high relapse rate in these patients after the first attack, which suggests that more positive treatment should be applied when these abnormal indicators are encountered to reduce relapse events in patients with NMOSD. Further studies with more data are needed to validate these conclusions.

DATA AVAILABILITY STATEMENT

The original contributions presented in 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 First Affiliated Hospital of Zhengzhou University (2019-KY-018). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL: methodology, formal analysis, data curation, writing — original draft, writing-review and editing. JZ: investigation, writing — review and editing. YZ: methodology, investigation, writing—review and editing. HX: investigation, writing — review and editing. RD and YY: formal analysis. LJ and JT: data curation. YJ: conceptualization, methodology, supervision, and funding acquisition. The first draft of the manuscript was written by YL. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.731835/full#supplementary-material

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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.

frontiers in Neurology

ORIGINAL RESEARCH
published: 09 September 2021
doi: 10.3389/fneur.2021.707046

Thyroid Function and Anti-thyroid Antibodies in Pediatric Anti-NMDAR Encephalitis

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

Received: *08 May 2021*
Accepted: *26 July 2021*
Published: *09 September 2021*

Citation:
Chen L, Wu W, Tian Y, Zeng Y, Hou C, Zhu H, Zheng K, Zhang Y, Gao Y, Peng B, Yang S, Wang X, Ning S, Liao Y, Lin H, Shi K, Li X and Chen W-X (2021) Thyroid Function and Anti-thyroid Antibodies in Pediatric Anti-NMDAR Encephalitis. Front. Neurol. 12:707046. doi: 10.3389/fneur.2021.707046

Objective: Recent studies found that changes of thyroid antibodies (ATAbs), thyroid hormone, and non-thyroidal illness syndrome (NTIS) characterized by thyroid hormone inactivation with low triiodothyronine and high reverse triiodothyronine followed by suppressed thyroid-stimulating hormone (TSH) in adult anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis were associated with disease severity. This study aimed to explore thyroid function and ATAbs in pediatric anti-NMDAR encephalitis and their clinical association.

Methods: We retrospectively analyzed the clinical data of 51 pediatric cases with anti-NMDAR encephalitis hospitalized in Guangzhou Women and Children's Medical Center from August 2016 to 2019.

Results: A percentage of 52.9% of patients belonged to the ATAb (+) group, with 26 cases both positive for anti-thyroid peroxidase antibodies (TPOAb) and anti-thyroglobulin antibodies (TGAb), and one patient only positive for TPOAb. A percentage of 62.7% of patients had at least one abnormality in terms of FT_3, free thyroxin (FT_4), or TSH levels. Meanwhile, 45.1% of patients were diagnosed with NTIS. Among 25 cases retested for thyroid function 2 months after the initial test, the respectively decreased FT_3 and FT_4 in 13 and 11 cases on admission returned to normal or closer normal than before; TPOAb in eight cases and TGAb in 12 cases were changed from positivity to negativity. Compared with onset, the level of TPOAb and TGAb at relapse remained stable or significantly decreased, respectively. Compared with the ATAb (–) group, the ATAb (+) group had an older onset age, a higher ratio of movement disorders, elevated rate of sleep disorders, increased anti-nuclear antibody positivity rate, and higher ratio of more than one course of intravenous immunoglobulin treatment. There were no significant differences between the NTIS and non-NTIS groups in clinical characteristics.

Conclusion: Anti-thyroid antibody positivity, abnormality of FT_3, FT_4, or TSH levels and NTIS are frequent in pediatric anti-NMDAR encephalitis. Thyroid antibody and thyroid hormone abnormalities could be improved through the course of treatment of

anti-NMDAR encephalitis. Cases with ATAbs (+) are at older onset ages and more likely to be treated by intravenous immunoglobulin therapy more than once. Unlike adult anti-NMDAR encephalitis, NTIS might not be associated with the clinical characteristics of anti-NMDAR encephalitis in pediatric patients.

Keywords: anti-NMDAR encephalitis, anti-thyroid antibody, thyroid hormone, non-thyroidal illness syndrome, children

INTRODUCTION

Anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis is an autoimmune disorder associated with autoantibodies binding with the NR1 subunit of the NMDAR receptor (1). The common clinical manifestations of anti-NMDAR encephalitis include psychiatric symptoms, behavioral dysfunction, seizures, movement disorder, speech disorder, cognitive impairment, decreased consciousness, autonomic dysfunction, or central hypoventilation (2). Anti-NMDAR encephalitis can be accompanied with other autoantibodies such as the myelin oligodendrocyte glycoprotein antibody (3), similar to other immune-mediated diseases, presenting more than one immune disorder together (4). Thyroid antibodies are frequently detected not only in patients with autoimmune thyroid disease but also in individuals without overt thyroid dysfunction, including those with rheumatoid arthritis, type 1 diabetes mellitus, Crohn's disease, and neurological disorders such as multiple sclerosis (5). Thyroid hormones are essential in humans. During brain development, thyroid hormones play an important role in the proliferation and differentiation of neuronal and glial progenitors (6, 7). In addition, hypothyroidism is associated with a decreased hippocampus size, which is a major area involved in anti-NMDAR encephalitis (1, 8). Studies have found that thyroid hormones affect the prognosis of critical and severe diseases, besides substance metabolism, growth, and development (9). Recent studies have reported thyroid antibody (10) and hormone (11) changes in adult anti-NMDAR encephalitis associated with disease severity. Non-thyroidal illness syndrome (NTIS) is characterized by thyroid hormone inactivation, with low triiodothyronine and high reverse triiodothyronine, followed by suppressed thyroid-stimulating hormone (TSH); NTIS is associated with clinical characteristics of adult anti-NMDAR encephalitis (11). However, few such reports are addressing pediatric anti-NMDAR encephalitis. In this study, we aimed to evaluate thyroid function and anti-thyroid antibodies in pediatric anti-NMDAR encephalitis.

Abbreviations: ATAbs, thyroid antibodies; NMDAR, N-methyl-D-aspartate receptor; ANA, anti-nuclear antibody; CSF, cerebrospinal fluid; EEG, electroencephalograph; FT_3, free triiodothyronine; FT_4, free thyroxin; IQR, interquartile range; IVIG, intravenous immunoglobulin; IVMP, intravenous methylprednisolone; MRI, brain magnetic resonance imaging; mRS, modified Rankin scale; NTIS, non-thyroidal illness syndrome; SSA, anti-Sjogren syndrome-related antigen A; TGAb, anti-thyroglobulin antibodies; TPOAb, anti-thyroid peroxidase antibodies; TSH, thyroid-stimulating hormone.

SUBJECTS AND METHODS

Subjects

Children with anti-NMDAR encephalitis were retrospectively recruited from August 2016 to August 2019 in the Department of Neurology of Guangzhou Women and Children's Medical Center. This study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center. Written and signed consent was obtained from the patients' parents or guardians, who also explicitly consented to publish their personal details, clinical data, and images that could identify them.

Methods

Inclusion Criteria

Patients aged younger than 18 years, diagnosed with anti-NMDAR encephalitis according to diagnostic criteria proposed by Graus et al. (2), and undergoing thyroid function tests including free triiodothyronine (FT_3), free thyroxin (FT_4), TSH, and anti-thyroid antibody (ATAb) tests including anti-thyroglobulin antibody (TGAb) and anti-thyroid peroxidase antibody (TPOAb) tests were involved.

Exclusion Criteria

Patients were excluded if they did not undergo thyroid hormone and thyroid function tests on admission, had known endocrine disorders or severe non-endocrine disorders that may influence thyroid function before anti-NMDAR encephalitis onset, or developed anti-NMDAR encephalitis after viral encephalitis.

Diagnostic Criteria of Hyperthyroidism, Subclinical Hyperthyroidism, and Non-thyroidal Illness Syndrome

Hyperthyroidism was diagnosed by endocrinologists according to the 2016 American Thyroid Association guidelines for diagnosis and management of hyperthyroidism (12). Subclinical hyperthyroidism was defined as a serum TSH concentration below the lower limit of the reference range, with serum FT_4 and FT_3 concentrations within their reference ranges. NTIS was characterized by the thyroid hormone inactivation with low triiodothyronine and high reverse triiodothyronine followed by suppressed TSH and was diagnosed based on serum FT_3 below the age-appropriate normal level and low or normal TSH level (13–18).

Clinical Data Collection

Clinical data of the involved patients, including age at onset, gender, clinical manifestations, prodromal infection, laboratory test results, electroencephalogram (EEG), brain magnetic resonance imaging (MRI), treatments, outcome, and follow-up, were reviewed. The modified Rankin scale (mRS) was

used to assess neurological disability at admission and discharge and at the end of follow-up (3, 19, 20). A poor response was defined as no mRS score improvement or mRS score ≥4 for 4 weeks (21). Relapse was defined as a new onset or worsening of symptoms occurring after at least 2 months of improvement or stabilization. The good long-term prognosis was defined as mRS score ≤2; the poor long-term prognosis was defined as mRS score >2 (21). Cerebrospinal fluid (CSF) pleocytosis was defined as a white blood cell (WBC) count > 15×10^6/l in our center. The movement disorders in anti-NMDAR encephalitis include orofacial, limb, or trunk dyskinesias (2).

Laboratory Measurement

All included patients underwent thyroid function and ATAb examinations upon admission, which were repeated 2 months later in some individuals. Thyroid function indexes, including FT_3, FT_4, and TSH levels, and ATAbs (TGAb and TPOAb) were measured using highly sensitive magnetic antibody enzyme-linked immunoassays. The normal range for both TPOAb and TGAb was 0–60 IU/ml in terms of laboratory standards. TPOAb positivity was defined as a TPOAb level higher than 60 IU/ml. TGAb positivity was defined as a TGAb higher than 60 IU/ml. According to ATAb results, a patient with TPOAb or TGAb positivity was assigned to the ATAb-positive group; otherwise, the case was assigned to the ATAb-negative group. The normal ranges of FT_3, FT_4, and TSH levels in children vary with age.

NMDAR IgG in both serum and CSF samples was determined by cell-based assays (EUROIMMUN, Lübeck, Germany) and CSF glial fibrillary acidic protein IgG and serum myelin oligodendrocyte glycoprotein IgG. These methods have been reported in detail in our previous study (22).

Treatment

First-line immunotherapy was performed with intravenous methylprednisolone (IVMP) combined with intravenous immunoglobulin (IVIG) in the acute phase. Two weeks after the first course of IVIG, if patients were not improved well, patients would receive the second course of IVIG treatment or second course of IVMP treatment. Second-line treatment included rituximab or cyclophosphamide administration. In addition, one patient diagnosed with hyperthyroidism was treated with methimazole.

Statistical Analysis

Statistical analysis was performed with IBM SPSS 20.0 for windows. Quantitative data with normal distribution were described as mean ± standard deviation and compared by Student's *t*-test or paired-sample *t*-test. Those with skewed distribution were presented as median with interquartile range (IQR) and compared by the Mann–Whitney *U*-test or Wilcoxon signed-rank test. Categorical data were described as frequency and percentage and compared by the chi-square test or Fisher's exact test. $p < 0.05$ (two-sided) was considered statistically significant.

RESULTS

Demographic and Clinical Characteristic Data

A total of 51 patients (male: female, 21:30) were enrolled, and their clinical features have been reported in our previous study (23). Onset age was 6.3 ± 2.7 years, ranging from 1.9 to 15 years. Prodromal infections occurred in 12 cases (23.5%, 12/51), including respiratory infection (11 cases) and urinary tract infection (1 case). Major symptoms in the whole treatment course were psychiatric symptoms (88.2%, 45/51), seizures (84.3%, 43/51), speech disorders (80.4%, 41/51), sleep disorder (72.5%, 37/51), abnormal movement (68.6%, 35/51), fever (51.0%, 26/51), decreased consciousness (39.2%, 20/51), and autonomic dysfunction (5.9%, 3/51). The median initial mRS score evaluated on admission was 4 (IQR, 3–4). One patient diagnosed with hyperthyroidism had emotional lability, heat intolerance, bad temper, profuse sweating, weight loss, and increased appetite, accompanied by second-degree thyroid enlargement without pain on palpation.

Ancillary Test Results

CSF Test

All patients underwent CSF examination after lumbar puncture in the acute phase. WBC count in the CSF at the first lumbar puncture was 44.4 ± 36.1×10^6/l, and CSF pleocytosis was observed in 41.2% (21/51) of patients. Protein elevation in the CSF (reference value, 0.15–0.45 g/l) was only detected in 5.9% (3/51) of patients, with amounts that ranged from 0.53 to 2.09 g/l. The levels of glucose and chloride were normal except in one patient who showed decreased glucose (2.35 mmol/l; the ratio of CSF glucose to finger stick glucose was 2.35/4.00 = 0.59). In this patient with reduced CSF glucose, CSF WBC was 90 × 10^6/l while the CSF protein level was within the normal reference range. These abnormalities of glucose level and WBC count in the CSF returned to normal after treatment with IVIG and IVMP, without antibiotic treatment. The CSF anti-NMDAR antibody was positive in all patients, while serum anti-NMDAR antibody was positive in 41.2% (21/51) of individuals.

ATAbs in Serum and Thyroid Function

On admission, all patients underwent examination of thyroid antibody tests, including serum TPOAb and TGAb. In total, five patients (9.8%, 5/51) received IVIG in other hospitals before thyroid antibody tests in our center. Among them, three patients received two courses of IVIG treatment, and two patients received one course of IVIG treatment. The median level of TPOAb and TGAb was 249.9 IU/ml (IQR, 48.0–454.7 IU/ml) and 78.6 IU/ml (IQR, 15.9–319.8 IU/ml), respectively. Twenty-seven cases (52.9%, 27/51) are TPOAb positive, and their median level of TPOAb was 373.7 IU/ml (IQR, 246.7–612.6 IU/ml). Twenty-six cases (51.0%, 26/51) are TGAb positive, of which the median level of 319.8 IU/ml (IQR, 183.8–499.4 IU/ml) and their TPOAb were positive as well. In total, 52.9% (27/51) of patients belonged to the ATAb (+) group, and 47.1% (24/51) were from the ATAb (–) group.

TABLE 1 | Results of thyroid hormone test on admission.

TSH/FT_3/FT_4	TSH (median, IQR, or mean ± SD, μIU/ml)	FT_3 (median, IQR, or mean ± SD, pmol/l)	FT_4 (median, IQR, or mean ± SD, pmol/L)	Number of patients (*n*1, %[a])	ATAb (+) (*n*2, %[b])	ATAb (–) (*n*3, %[c])
NNN	2.116 (1.098, 2.885)	5.14 (4.84, 5.71)	19.1 (18.27, 20.18)	19 (37.3%)	9 (47.4%)	10 (52.6%)
NN↑	2.119 ± 0.829	5.57 ± 1.15	24.23 ± 0.88	3 (5.9%)	2 (66.7%)	1 (33.3%)
NN↓	1.261 ± 0.490	5.36 ± 0.42	14.37 ± 1.29	2 (3.9%)	1 (50.0%)	1 (50.0%)
N↓N	1.862 ± 0.651	5.35 ± 1.72	18.27 ± 0.53	3 (5.9%)	0 (0.0%)	3 (100.0%)
N↓↓	1.673 (1.286, 2.312)	3.29 (3.23, 3.44)	12.72 (11.96, 13.51)	4 (7.8%)	3 (75.0%)	1 (25.0%)
↓↓↓	1.219 (0.687, 2.055)	4.73 (3.38, 5.87)	18.58 (13.06, 20.46)	13 (25.5%)	9 (69.2%)	4 (30.8%)
↓↓N	0.450 ± 0.203	3.78 ± 0.26	17.20 ± 1.71	3 (5.9%)	2 (66.7%)	1 (33.3%)
↓NN	0.251 ± 0.125	5.38 ± 1.06	22.70 ± 3.53	3 (5.9%)	0 (0.0%)	3 (100.0%)
↓↑↑	0.001	94.3	>30.8	1 (2.0%)	1 (100.0%)	0 (0.0%)
Total	1.420 (0.841, 2.344)	4.91 (4.05, 5.71)	18.78 (16.93–20.40)	51 (100%)	27 (52.9%)	24 (47.1%)

N, normal; ↓, decrease; ↑, increase; [a]n1/51 × 100%; [b]n2/n1 × 100%; [c]n3/n1 × 100%; FT_3, free triiodothyronine; FT_4, free thyroxin; TSH, thyroid-stimulating hormone.

On admission, all included patients underwent examinations for serum FT_3, FT_4, and TSH. The median levels of FT_3, FT_4, and TSH were 4.91 pmol/l (IQR, 4.04–5.71 pmol/l), 18.78 pmol/l (IQR, 16.93–20.40 pmol/l), and 1.420 μIU/ml (IQR, 0.841–2.344 μIU/ml), respectively. Totally, 62.7% (32/51) of patients had at least one abnormality of FT_3, FT_4, or TSH level compared with age-appropriate normal levels. Abnormal FT_3 was seen in 47.1% (24/51) of patients, including FT_3 reduction (95.8%, 23/24) and FT_3 elevation (4.2%, 1/24). Abnormal FT_4 was found in 45.1% (23/51) of patients, including FT_4 decrease (82.6%, 19/23) and FT_4 elevation (17.4%, 4/23). Decreased TSH as an abnormality was observed in 39.2% (20/51) of patients. Combining FT_3, FT_4, and TSH to classify these abnormalities (more details in **Table 1**), patients with concurrently decreased FT_3, FT_4, and TSH (40.6%, 13/32) were the most frequent group, followed by those with decreases in both FT_3 and FT_4 but normal TSH (12.5%, 4/32). Decreased TSH and normal FT_3 and FT_4 levels were only found in three patients (9.4%, 3/32) who did not show the clinical presentations of hyperthyroidism. However, the thyroid function test in these three patients showed that TSH levels returned to normal at 2 months after the first test; therefore, these three patients did not meet the criteria for diagnosing subclinical hyperthyroidism. Increased FT_3 and FT_4 amounts and decreased TSH levels were only detected in one patient diagnosed with hyperthyroidism. According to the NTIS diagnosis criteria, 45.1% (23/51) of patients were diagnosed with NTIS, while 54.9% (28/51) did not have NTIS. The 28 patients with no NTIS included 19 individuals with normal FT_3, FT_4, and TSH levels, three cases with decreased TSH and normal FT_3 and FT_4 levels, three cases with increased FT_4 and normal FT_3 and TSH levels, two cases with decreased FT_4 and normal FT_3 and TSH levels, and the remaining one patient diagnosed with hyperthyroidism.

Two months after the first test, 49.0% (25/51) of patients were reexamined for thyroid function and ATAbs, including 13 out of 23 cases with initially decreased FT_3, 11 out of 19 cases with decreased FT_4, 18 out of 27 cases with TPOAb positivity, and 17 out of 26 cases with TGAb positivity. The results showed that FT_3 levels in all 13 patients with initially decreased FT_3 significantly returned to the normal range or maintained higher than before (6.1 ± 1.9 pmol/l vs. 4.7 ± 1.4 pmol/l; Mann–Whitney *U*-test, $Z = -2.866$, $p < 0.01$); FT_4 levels in all 11 patients with initially decreased FT_4 were also significantly increased to the normal range (21.5 ± 5.3 pmol/l vs. 18.1±4.4 pmol/l; Mann–Whitney *U*-test, $Z = -2.597$, $p < 0.01$). TPOAb amounts in all 18 patients with initial TPOAb positivity significantly decreased from 446.2 IU/ml (IQR, 243.6–670.1 IU/ml) to 85.3 IU/ml (IQR, 42.9–225 IU/ml) (Mann–Whitney *U*-test, $Z = -3.101$, $p < 0.01$); TPOAb levels in eight of these 18 patients (44.4%, 8/18) became negative. TGAb amounts in the 17 patients with initial TGAb positivity significantly decreased from 211.5 IU/ml (IQR, 146.9–337.3 IU/ml) to 24.9 IU/ml (IQR, 17.3–73.5 IU/ml) (Mann–Whitney *U*-test, $Z = -2.343$, $p = 0.02$); TGAb levels in 12 of these 17 patients (70.6%, 12/17) became negative. Only one patient, diagnosed with hyperthyroidism, had repeated evaluation of ATAbs and thyroid function 4 months after treatment with methimazole, and the results showed that the increased levels of TSH from 0.001 to 0.023 μIU/ml, decreased levels of FT_3 from >30.8 to 7.44 pmol/l, normalized levels of FT_4 from 94.3 pmol/l, and decreased levels of TGAb from >500 to 393.2 IU/ml were revealed; TPOAb levels both on admission and reexamination were >1,300 IU/ml.

Serum Pathogenic Test

All patients underwent pathogenic serum tests including IgM antibodies for human Chlamydia psittaci, Mycoplasma pneumonia, Legionella pneumonia type 1, Coxiella burnetii, respiratory adenovirus, influenza A, influenza B, parainfluenza, respiratory syncytial virus, and herpes simplex virus. The results showed positive outcomes in the 13 cases (25.5%, 13/51), including eight cases that are serum Herpes simplex virus IgM positive and five cases that are mycoplasma pneumonia IgM positive, respectively.

Other Coexisting Autoantibodies in Serum and CSF

Totally, 51 patients underwent a coexisting autoantibody test for serum and CSF samples. Except for anti-NMDAR antibody and ATAbs, the other autoantibodies tested included

IgG antibodies for glutamate and γ-aminobutyric acid alpha and beta receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor1, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor2, leucine-rich glioma-inactivated 1 protein, and contactin-associated protein-like 2 in CSF and serum, IgG antibodies for myelin oligodendrocyte glycoprotein and aquaporin-4 in serum, and antinuclear antibody, anti-nuclear ribonucleoprotein antibody, anti-Sjogren syndrome-related antigen A (SSA)/52 KD, SSA/60 KD, and anti-Sjogren syndrome-related antigen B in serum. A percentage of 21.6% (11/51) of patients showed positivity for other autoantibodies. Only anti-nuclear antibody (ANA) positivity was seen in seven cases (13.7%, 7/51), and only SSA positivity in two cases (3.9%, 2/51); both ANA and SSA positivities were detected in 2 cases (3.9%, 2/51). Myelin oligodendrocyte glycoprotein antibody positivity was seen in one patient.

EEG

All patients underwent EEG. Totally 49 cases had abnormal EEG, including 88.2% (45/51) with slow wave, 35.3% (18/51) with epileptic activity, and 2.0% (1/51) with Delta brush.

Imaging Examination

All patients underwent brain MRI examination in the acute phase, and 15.7% (8/51) had parenchymal lesions, with the most common location being the temporal lobe (five cases), followed by the insular lobe (four cases). Totally three patients underwent ultrasound examination of the thyroid: two cases had a normal organ, while a patient diagnosed with hyperthyroidism showed diffuse goiter and thyroid inferno. All patients underwent tumor screening, including chest computer tomography scans and abdominal and genital MRI scans. No patients had tumors.

Treatment

All patients were treated by first-line immunotherapy with IVMP combination with IVIG. Thirty-two patients received two courses of IVIG treatment, and 19 patients received only one course of IVIG treatment. Forty-nine patients received one course of IVMP treatment, and only two patients received two courses of IVMP treatment. Totally, 78.4% (40/51) of patients had a good response to the first-line therapy. The remaining 11 patients had poor response to the first-line immunotherapy, among whom six cases were subsequently treated with rituximab as second-line immunotherapy and improved without relapse; five patients continued treatment with IVIG monthly (a total of 400 mg/kg/day monthly) and a low oral dose of corticosteroid treatment (0.5–1 mg/kg/day of prednisone for 1–2 weeks, with dosage tapering weekly for 3–6 months). Totally, three out of these five patients, who continued to be treated with IVIG and a low oral dose of corticosteroid, improved without relapse, while the remaining two cases relapsed and were further treated with cyclophosphamide. Consequently, after treatment with cyclophosphamide, these two patients improved without relapse.

In addition, 15 cases were treated with antiepileptic drugs—of these, nine and six patients with two types of antiepileptic drugs for seizures' control needed.

One patient diagnosed with hyperthyroidism was treated with methimazole and a low-iodine diet.

As supportive treatment, one patient received tracheal intubation and mechanical ventilation. Totally three cases accompanied by infection received antibiotic therapy, including meropenem (two cases) and a combination of vancomycin and cefoperazone-sulbactam (one case). In addition, nine cases were treated with risperidone to improve the abnormal mental behaviors.

Course and Relapse

The median length of the first hospital stay was 28.8 ± 10.2 days. Seven patients (13.7%, 7/51) relapsed during corticosteroid weaning (three cases) or after withdrawal (four cases). Compared with TPOAb levels at onset, TPOAb amounts on relapse tended to decrease without statistical significance [341.8 IU/ml (IQR, 110.6–902.4 IU/ml) at onset vs. 34.6 IU/ml (IQR, 30.2–145.9 IU/ml) at relapse; Wilcoxon signed-rank test, $p = 0.07$]. In addition, compared with TGAb amounts at onset, TGAb levels on relapse were significantly decreased [295.8 IU/ml (IQR, 78.2–482.4 IU/ml) at onset vs. 16.6 IU/ml (IQR, 15–23.45 IU/ml) at relapse; paired-sample t-test, $p = 0.02$].

Prognosis

One patient (2.0%) was lost to follow-up for poor compliance. The duration of follow-up was 20.0 ± 6.7 months. The median initial mRS score in the 50 followed patients was 4 (IQR, 3–4), which was significantly higher than the value at the last follow-up of 0 (IQR, 0–1) ($Z = -6.386$, Wilcoxon signed-rank test, $p < 0.01$). All the followed-up patients were alive at the last follow-up and achieved a good prognosis (mRS ≤ 2).

Clinical Comparison Between ATAb (+) and ATAb (–) Groups

There was no significant difference in gender distribution between the ATAb (+) and ATAb (–) groups, whereas onset age was elevated in the ATAb (+) group compared with the ATAb (–) group (7.1 ± 2.9 vs. 5.3 ± 2.2 years, $p = 0.02$). As for clinical characteristics, compared with the ATAb (–) group, the ATAb (+) group had higher rates of movement disorders (88.9 vs. 45.8%, $p < 0.01$) and sleep disorders (85.2 vs. 58.3%, $p = 0.03$), ANA positivity (29.6 vs. 4.1%, $p = 0.03$), and IVIG treatment ≥2 courses (77.8 vs. 45.8%, $p = 0.02$). Details are shown in **Table 2**.

Clinical Comparison Between Patients With and Without NTIS

Patients with anti-NMDAR encephalitis were divided into the NTIS and non-NTIS groups based on serum FT_3 and TSH amounts. There were no significant differences between these two groups in gender distribution, onset age, clinical symptoms, and rates of prodromal infections, pleocytosis in CSF, ANA positivity, lesions in brain MRI, IVIG treatment ≥2 courses and treatment with rituximab, and mRS score on admission or at first discharge and relapse rate (more details in **Table 3**).

TABLE 2 | Comparison of clinical characteristics between the ATAb (+) and ATAb (–) groups.

	ATAbs (+) $n = 27$	ATAbs (–) $n = 24$	*p*
Male/female	12/15	9/15	0.62[a]
Age (mean ± SD,years)	7.1 ± 2.9	5.3 ± 2.2	0.02[b]
Fever, *n* (%)	15 (55.6)	11 (45.8)	0.49[a]
Decrease consciousness, *n* (%)	13 (48.1)	7 (29.1)	0.49[a]
Movement disorders, *n* (%)	24 (88.9)	11 (45.8)	<0.01[a]
Epileptic seizures, *n* (%)	22 (81.4)	21 (87.5)	0.71[c]
Speech disorders, *n* (%)	24 (88.9)	17 (70.8)	0.16[c]
Psychiatric symptoms, *n* (%)	26 (96.3)	19 (79.1)	0.09[c]
Sleep disorders, *n* (%)	23 (85.2)	14 (58.3)	0.03[a]
Prodromal infections, *n* (%)	9 (33.3)	3 (12.5)	0.08[a]
HSV IgM (+) *n* (%)	4 (14.8)	4 (16.7)	1.00[c]
Pleocytosis in CSF, *n* (%)	12 (44.4)	9 (37.5)	0.62[a]
ANA positive, *n* (%)	8 (29.6)	1 (4.1)	0.03[c]
Lesions in brain MRI, *n* (%)	4 (14.8)	4 (16.7)	1.00[c]
IVIG (≥2 courses), *n* (%)	21 (77.8)	11 (45.8)	0.02[a]
Rituximab, *n* (%)	4 (14.8)	2 (8.3)	0.67[c]
Hospital day (d)	31.0 ± 9.9	26.1 ± 9.6	0.06[d]
mRS on admission	4 (3, 4)	4 (3, 4)	0.40[d]
mRS at discharge	2 (2, 3)	2 (2, 3)	0.55[d]
Relapse, *n* (%)	5 (18.5)	2 (8.3)	0.43[c]

[a]*Chi-square test;* [b]*independent t-test;* [c]*Fisher's exact test;* [d]*Mann–Whitney U-test. ANA, antinuclear antibody; CSF, cerebrospinal fluid; HSV, Herpes simplex virus; IVIG, intravenous immunoglobulin; MRI, magnetic resonance imaging; mRS, modified Rankin scale; SD, standard deviation.*

TABLE 3 | Comparison of clinical characteristics between NTIS and not NTIS group.

	NTIS $n = 23$	Not NTIS $n = 28$	*p*
Male/female	9/14	12/16	0.79[a]
Age (mean ± SD, years)	6.5 ± 2.7	6.0 ± 2.7	0.66[b]
Fever, *n* (%)	9 (39.1)	17 (60.7)	0.13[a]
Decrease consciousness, *n* (%)	10 (43.5)	10 (35.7)	0.57[a]
Movement disorders, *n* (%)	15 (65.2)	20 (71.4)	0.63[a]
Epileptic seizures, *n* (%)	18 (78.3)	25 (89.3)	0.28[c]
Speech disorders, *n* (%)	18 (78.3)	23 (82.1)	0.74[c]
Psychiatric symptoms, *n* (%)	21 (91.3)	24 (85.7)	0.68[c]
Sleep disorders, *n* (%)	16 (69.6)	21 (75.0)	0.67[a]
Prodromal infections, *n* (%)	6 (26.1)	6 (21.4)	0.70[a]
Pleocytosis in CSF, *n* (%)	7 (30.4)	14 (50.0)	0.16[c]
ANA positive, *n* (%)	3 (13.0)	6 (21.4)	0.48[c]
Lesions in brain MRI, *n* (%)	6 (26.1)	2 (7.1)	0.12[c]
IVIG (≥2 courses), *n* (%)	15 (65.2)	17 (60.7)	0.95[a]
Rituximab, *n* (%)	3 (13.0)	3 (10.7)	1.00[c]
Hospital day (d)	27.3 ± 10.2	29.8 ± 9.8	0.32[d]
mRS on admission	4 (3.4)	4 (4.4)	0.92 [d]
mRS at discharge	3 (2.4)	2 (1.3)	0.10[d]
Relapse, *n* (%)	2 (8.7)	5 (17.9)	0.44[c]

[a]*Chi-square test;* [b]*independent t-test;* [c]*Fisher's exact test;* [d]*Mann–Whitney U-test. ANA, antinuclear antibody; CSF, cerebrospinal fluid; IVIG, intravenous immunoglobulin; MRI, magnetic resonance imaging; mRS, modified Rankin Scale; SD, standard deviation.*

DISCUSSION

In the present study, we aimed to evaluate thyroid function and ATAbs in pediatric anti-NMDAR encephalitis. We found that 52.9% of patients had TPOAb positivity, and 51.0% had TGAb positivity. Totally 52.9% of patients belonged to the ATAb (+) group. ATAbs also occur in immune-mediated nervous diseases such as multiple sclerosis (24), optic neuromyelitis (25, 26), limbic encephalitis (27), Hashimoto's encephalopathy (28), and adult anti-NMDAR encephalitis (10). ATAbs in adult anti-NMDAR encephalitis were reported in 52.4% of cases, corroborating the current findings (10). The prevalence rates of ATAbs in a study of adult anti-NMDAR encephalitis and ours were distinctly higher than those of 10–15% found in the general population (27, 28). Besides, we also found that patients in the ATAb (+) group had partly different characteristics than the ATAb (–) group. The ATAb (+) group at older onset age had a higher rate of movement and sleep disorders, had elevated frequencies of ANA positivity, needed more courses of IVIG treatment, and had longer hospital stays. Although in the previous report, the adult patients with anti-NMDAR encephalitis in the ATAb (+) group also showed different clinical characteristics from the ATAb (–) group, and those differences were not the same findings in the current work (10). Specifically, the adult patients with anti-NMDAR encephalitis in the ATAb (+) group had a higher mRS score at admission or discharge, a higher rate of epileptic seizures and consciousness disorder, and an increased rate of lesions in brain MRI. However, the adult study did not compare the difference in the rates of movement disorders and sleep disorders, ANA positivity, and the course of IVIG treatment between the ATAb (+) and ATAb (–) groups (10). In addition, we also found no differences in the rates of seizures, conscious disorder, and lesions in brain MRI between the ATAb (+) and ATAb (–) groups. The difference between this trial and the adult study might be caused by the difference of study subjects or the limited sample size. Although there were differences between the ATAb (+) and ATAb (–) groups, it is unclear whether ATAbs play a direct pathogenic role in anti-NMDAR encephalitis. It was reported that thyroid globulin might cross-react with myelin-related antigen epitopes in molecular simulation, indicating that TGAb could cause brain injury (24). In addition, TPOAb could specifically bind to cerebellar astrocytes in patients with Hashimoto's encephalopathy (29). It was speculated that TPOAb might affect glial function after interacting with glial cells, thus resulting in neuronal dysfunction (29). However, in this study, ATAbs were not associated with disease severity at onset and the level of TGAb decreased at relapse, and TPOAb remained stable at relapse. We speculated that elevated ATAbs might be associated with increased hyperactivity immune response at the onset of anti-NMDAR encephalitis in the ATAb (+) group, and these

patients required more courses of IVIG treatment. However, anti-NMDAR encephalitis relapse was not associated with elevated ATAbs in this study.

We found that 62.7% of patients had at least one abnormality of FT_3, FT_4, or TSH levels compared with age-appropriate normal values. Abnormalities of FT_3, FT_4, and TSH were found in 47.1%, 45.1%, and 39.2% of cases, respectively. In addition, 45.1% (23/51) of patients were diagnosed with NTIS, also known as low triiodothyronine syndrome, and could be caused by some pathologies in the absence of thyroid disease (30). NTIS occurs in many hospitalized patients, especially critically ill patients in the ICU (31, 32). NTIS has also been reported in neuroimmune diseases, including anti-NMDAR encephalitis (11, 24–26). Studies showed that adult patients with anti-NMDAR encephalitis with NTIS have a higher rate of decreased consciousness, an elevated mRS score at admission, and longer hospital stay (11). However, in this study, no significant differences were found in clinical symptoms, mRS scores at admission or discharge, and hospital stay between the NTIS and non-NTIS groups. The inconsistency between the adult trial and this study may be caused by differences in study subjects. In addition, we could not fully confirm that NTIS had no effects on pediatric anti-NMDAR encephalitis due to the small sample size and only a single time point at admission for the thyroid function tests. NTIS could occur later after hospital admission, so some possible NTIS cases might not have been detected, which might affect the finding that NTIS was not associated with clinical features. Further investigation with large sample size and multiple time point tests for the thyroid function tests is required.

In NTIS, thyroid hormone treatment is controversial. Some researchers believed that proper thyroid hormone therapy is beneficial to disease rehabilitation (30). However, the benefit of thyroid hormone treatment is not always achieved (33, 34). A double-blind randomized placebo-controlled study of children with NTIS after cardiac surgery found that thyroid hormone treatment failed to improve growth and neurodevelopment after long-term observation (33). Meanwhile, others disagreed with thyroid hormone treatment in NTIS patients and considered that NTIS compensatorily responded to diseases and aggressive treatment might be counterproductive (9). The present study did not perform thyroid hormone treatment for NTIS. Nevertheless, FT_3, FT_4, or TSH levels returned to normal or significantly increased, accompanied by improved anti-NMDAR encephalitis. Actually, NTIS may be related to variations of inflammatory factors (30). After primary disease improvement, inflammation in the primary disease could be controlled, and NTIS would also be improved. Therefore, we hypothesized that NTIS in pediatric anti-NMDAR encephalitis is a temporary change in an acute phase of the disease, which requires follow-up and can recover without thyroid hormone treatment.

Our study has several limitations. The first limitation is that only a single time point at admission for the thyroid function tests may affect the finding that NTIS was not associated with clinical features. The second limitation is that our study is a retrospective study and lacks follow-up thyroid antibody and thyroid function testing in around half of the subjects. In addition, in terms of the ANA antibody test, ANA positivity was only seen in a total of nine cases, among whom only two patients underwent the concentration of the ANA antibody test (33.32 and 90.29 IU/l, respectively). For the limitation of the retrospectively study, we could not provide the ANA values for the other seven patients.

CONCLUSION

Anti-thyroid antibody positivity, abnormality of FT_3, FT_4, or TSH levels and NTIS are frequent in pediatric anti-NMDAR encephalitis. Thyroid antibody and thyroid hormone abnormalities could be improved through the course of treatment of anti-NMDAR encephalitis. Cases with ATAbs (+) at older onset ages are more likely to be treated by intravenous immunoglobulin therapy more than once. Unlike adult anti-NMDAR encephalitis, NTIS might not be associated with the clinical characteristics of anti-NMDAR encephalitis in pediatric patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center (2019052419364384). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LC, WW, YT, and YiZ study concept, acquisition of data, and drafting of the manuscript. CH, HZ, KZ, YaZ, and YG study concept and acquisition of data. BP, SY, XW, SN, YL, HL, and KS analysis of data and interpretation. XL and W-XC study concept and critical revision of the manuscript for intellectual content. All the authors gave final approval of the version to be published.

FUNDING

This present study was supported by the Health and Family Planning Technological Project Foundation of Guangzhou City (Grant No. 20181A011038). The funders had no role in the study concept, study design, data analysis, interpretation, or reporting of the results. The authors had full control of the data and information submitted for publication.

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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.

frontiers in Neurology

ORIGINAL RESEARCH
published: 22 September 2021
doi: 10.3389/fneur.2021.729075

CRMP5 Antibodies—Diagnostic Challenges

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CRMP5-associated paraneoplastic neurological syndromes (PNS) are rare, and only few studies describe larger cohorts of patients with CRMP5 antibodies. We have included 24 patients with CRMP5 antibodies and compared clinical findings with diagnostic findings from two different line assays (Ravo and Euroimmun), staining of cerebellar sections and results of a newly developed cell-based assay for detection of CRMP5 antibodies, CRMP5-CBA. We found that peripheral neuropathy and cerebellar ataxia together with lung cancer were the most common diagnoses associated with CRMP5 antibodies. CRMP5-CBA was easy to perform, identified all relevant cases for CRMP5-associated PNS and is therefore a valuable add-on for verification of CRMP5 positivity in diagnosis of PNS.

Keywords: paraneoplastic neurological disease, CRMP5 antibody, peripheral neuropathy, cerebellar ataxia, lung cancer

OPEN ACCESS

Edited by:
Scott Douglas Newsome, Johns Hopkins Medicine, United States

Reviewed by:
Michael Davin Kornberg, The Johns Hopkins Hospital, United States
Shuhei Nishiyama, Massachusetts General Hospital and Harvard Medical School, United States

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

Received: *22 June 2021*
Accepted: *23 August 2021*
Published: *22 September 2021*

Citation:
Totland C, Haugen M and Vedeler C (2021) CRMP5 Antibodies—Diagnostic Challenges. Front. Neurol. 12:729075. doi: 10.3389/fneur.2021.729075

INTRODUCTION

CRMP5 antibodies were first described by Honnorat et al. who called them anti-CV2 (1). These antibodies were found in sera from some patients with paraneoplastic neurological syndromes (PNS) and stained the cytoplasm and processes of oligodendrocytes in the brain stem, spinal cord and cerebellar white matter (1). The antigen was later identified as collapsin response mediator protein 5 (CRMP5), a protein involved in neurite development (2).

PNS commonly associated with CRMP5 antibodies include Lambert-Eaton myasthenic syndrome, limbic encephalitis, encephalomyelitis, cerebellar ataxic syndrome and peripheral neuropathy (1, 3, 4). An underlying cancer can be identified in about 73% of patients with CRMP5 antibody associated PNS (5), and CRMP5 antibodies often coexist with other paraneoplastic antibodies, most commonly anti-Hu (3, 4).

Lung cancer, especially small cell lung cancer (SCLC), and thymoma are the most frequent malignancies found in patients with CRMP5 antibodies (3, 4, 6, 7). CRMP5 is universally expressed in SCLC (6) and CRMP5 antibodies have also been identified in ∼5% of the patients with SCLC without PNS (8). Further, 12% of all patients with thymoma and myasthenia gravis have CRMP5 antibodies (8), even though CRMP5 expression has not been found in thymus or thymoma either in patients with CRMP5 antibodies or those without (5).

Immunohistochemical staining with patient sera on fixed rat cerebellar tissue or commercial line assays are the preferred techniques for detection of CRMP5 antibodies. A positive finding in one test should be confirmed by another test and compared with clinical findings before a diagnosis is set. That there are currently only two valid ways to detect CRMP5 antibodies represents several problems. Firstly, CRMP5 antibodies are best detected on rat cerebellar tissue from rats transcardiacally perfused with paraformaldehyde (PFA), and further post fixation of cerebellum in PFA (1). This technique can be challenging to perform at many diagnostics laboratories, as not

all have proper animal facilities for such methods. Secondly, commercial available line assays are easier to perform, but recent studies have highlighted that these assays often pick up to many false positives. For CRMP5 it has been estimated that about 50% of all positive findings are false positive (9, 10), so an easy to perform validation assay is much needed.

METHODS

Patient Selection

In the period 2003–2021, 35,553 patient sera and cerebrospinal fluid (CSF) samples were analyzed for paraneoplastic antibodies at the Neurological Research Laboratory, Haukeland University Hospital, Bergen, Norway. Of these, 36 sera/CSF (24 patients) were positive for CRMP5 antibodies on the 14 PNS line assay from Ravo Diagnostika and were included in this study. These samples were further analyzed using EUROLINE PNS 12 Ag, by indirect immunofluorescence on rat cerebellar sections, and by a newly developed cell-based assay for detection of CRMP5 antibodies (CRMP5-CBA) produced by Euroimmun. Clinical data were obtained from referring neurologists. The study was approved by the regional ethics committee (#242339) as a quality assessment study.

Line Assay

Two commercially available line assays were used for initial screening for onconeural antibodies. The PNS 14 Line Assay (Ravo Diagnostika, #PNS14-003) includes 14 different antigens for PNS: GAD65, HuD, Yo, Ri, CV2/CRMP5, amphiphysin, Ma1, Ma2, SOX1, Tr/DNER, Zic4, titin, recoverin and Protein Kinase C γ. The EUROLINE PNS 12 Ag (Euroimmun, #DL1111-1601-7-G) includes 12 different antigens for PNS: amphiphysin, CV2/CRMP5, Ma2, Ri, Yo, Hu, recoverin, SOX1, titin, Zic4, GAD65 and Tr/DNER. Serum and CSF samples from 24 patients were analyzed in both line assays following the manufacturer's instructions. Two independent investigators graded band intensities from + (weakly positive) to + + + (strongly positive), compared to a positive control sample (+ + +).

Indirect Immunofluorescence on Rat Cerebellar Sections

Wistar Hannover GLAST rats were anesthetized and transcardiacally perfused with ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed (24 h, 4°C) in PFA, then incubated with 18% sucrose in PBS (72 h, 4°C), snap-frozen, and cut into 10-μm parasagittal sections on a cryostat. Heat-induced antigen retrieval was performed in a 2100 Antigen retriever in Diva Decloaker buffer solution (Biocare Medical, #DV2004MX). Sections were blocked in 0,2% bovine serum albumin (BSA) and 1% Triton X-100 in PBS (2 h, room temperature) and incubated over night at 4 °C with patient samples diluted 1:500 and rabbit-anti-CRMP5 (1:200, Abcam, #AB36203) in blocking solution. The sections were then washed with PBS and incubated with secondary antibody (Alexa Fluor® 488 goat anti-human IgG, Thermo Fisher Scientific, #A-11013, and Alexa Fluor® 594 goat anti-rabbit, Thermo Fisher Scientific, #A11012) diluted 1:100 in blocking buffer for 90 min at room temperature. Sections were then washed in PBS and mounted with Fluoromount-G (Thermo Fisher Scientific, #00-4958-02) and examined by immunofluorescence. Two independent investigators evaluated the results. All procedures were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals Norway (FOTS 20135149/20157494/20170001).

CRMP5 Cell-Based Assay

Anti-CV2/CRMP5 IIFT (#FA 1119-1010-51, Euroimmun) is a test kit from Euroimmun that is not commercially available yet. It is a cell-based assay with HEK293 cells transfected with CRMP5. The kit consist of slides, and each slide contains 10 biochips. Each chip has one field with transfected cells and one field with untransfected cells. The kit was used according to the manufacturer's instructions. Briefly, serum samples were diluted 1:10 and 1:100 in sample buffer. When only CSF was available, this was tested undiluted 1:1. Sample (30 μl) was added to each biochip and incubated at room temperature for 30 min. To verify that serum stained only the CRMP5-positive cells, a co-staining with the rabbit anti-CRMP5 antibody was performed. Slides were then washed with phosphate-buffered saline containing 0.2% Tween 20 (PBS-Tween 20) for 5 min at room temperature, before incubation with secondary antibody (Alexa Fluor 488 goat anti-human IgG and Alexa Fluor 594 goat-anti rabbit (1:500, 30 min, RT). Slides were rinsed with PBS-Tween 20, and mounted on a glass coverslip. The cut-off for anti-CV2/anti-CRMP5 was set to 1:100, as advised by the manufacturer. Sera from 25 CRMP5 negative patients were included as negative controls. Two independent investigators evaluated the results.

RESULTS

Clinical and diagnostic findings of the 24 patients (17 females and 7 males, mean age 67 years) are presented in **Table 1**. Lung cancer was the cancer most frequently associated with CRMP5 antibodies (14 patients, 58%) and peripheral neuropathy (sensory-motor neuropathy) was the most prevalent neurological diagnosis (10 patients, 42%) associated with such cancer. Cerebellar ataxia was diagnosed in three patients with lung cancer and one patient with lymphoma. One patient had thymoma and myasthenia gravis, and one patient had neuroendocrine carcinoma and encephalitis. No tumor was found in seven patients (30%) that tested positive for CRMP5 antibodies by Ravo line assay. These patients showed a broader spectrum of neurological diseases, including peripheral neuropathy, cerebellar ataxia, cranial neuropathy, brain infarction, myalgia and radiological isolated syndrome.

In 14 patients (58%) CRMP5 antibodies were the only antibodies identified by the Ravo line assay, while in the other 10 patients (42%) additional antibodies were found. The most common antibodies co-expressed with CRMP5 according to the Ravo line assay were Hu (5 patients, 21%), SOX1 (4 patients, 17%), amphiphysin (2 patient, 8%) and Ri (1 patient, 4%). In two of the patients (8%), both Hu and Sox1 were co-expressed with CRMP5. There were some small differences in the prevalence

TABLE 1 | Diagnostic and clinical findings in 24 patients with CRMP5 antibodies.

Nr	Sex, age	Line assay RAVO		Lineassay Euroimmun		Cerebellar sections	CBA	Neurological symptoms	Cancer
		CRMP5	*Other AB*	*CRMP5*	*Other AB*				
1	M, 68	+++		+++		+	1/100	Peripheral neuropathy	Lung cancer
2	F, 62	+		++		+	1/10,000	Peripheral neuropathy	Lung cancer
3	F, 75	++	Hu/Sox1	++	Hu/Sox1	+	1/10,000	Peripheral neuropathy	Lung cancer
4	F, 71	++	Sox1	-	Amph	-	1/10,000	Peripheral neuropathy	Lung cancer
5	F, 68	++	Hu	++	Hu/Zic4	+	1/100,000	Peripheral neuropathy	Lung cancer
6	F, 78	+++	Amph	+++	Amph	+	1/100,000	Peripheral neuropathy	Lung cancer
7	F, 74	+++		+++		+	1/100,000	Peripheral neuropathy	Lung cancer
8	M, 68	++		+		-	1/100,000	Peripheral neuropathy	Lung cancer
9	F, 61	++	Sox1	++	Sox1	+	1/10,000	Peripheral neuropathy	Lung cancer
10*	F, 78	+++		++	Recoverin	-	1/10,000	Peripheral neuropathy	Lung cancer
11	F, 70	++	Hu	+	Hu	-	1/100,000	Optic neuritis	Lung cancer
12*	F, 76	+++		++		+	1/1,000	Cerebellar ataxia	Lung cancer
13	M, 70	+++	Amph	++	Amph	+	1/10,000	Cerebellar ataxia	Lung cancer
14	F, 69	+++	Hu	+++	Hu	+	1/100,000	Cerebellar ataxia	Lung cancer
15*	F, 68	++		+		+	1/10,000	Cerebellar ataxia	Lymphoma
16	M, 86	+	Ri	+	Sox1/Ri	-	1/100,000	Encephalitis	Neuroendocrine carcinoma
17	F, 60	++		+++		+	1/1,000	Myastenia gravis	Thymoma
18	F, 84	++		++		+	1/100,000	Cerebellar ataxia	No
19	M, 66	+++	Hu/Sox1	+++	Hu/Recoverin	+	1/10,000	Peripheral neuropathy	No
20	F, 43	+		-		-	1/100	Radiological isolated syndrome	No
21*	M, 71	++		-		-	1/10	Brain infarction	No
22	K, 23	++		++		-	-	Brain infarction	No
23	F, 69	+		+		-	-	Cranial neuropathy	No
24	M, 61	+		-		-	-	Myalgia	No

*, *CSF. +-+++, intensity score for line immunoassays; +, weakly positive; ++, medium positive; +++, strongly positive. Immunostaining of cerebellar sections is markerd as + (positive) or – (negative). Titers are indicated for the cell-based assay (CBA), while negative staining is indicated with -. Amph, Amphiphysin. Peripheral neuropathy = sensorimotor polyneuropathy. Follow-up of the cancer negative patients was > 2 years except for patient #21 and #22 with 1 year follow-up.*

of additional antibodies identified by Ravo and Euroimmun line assays (**Table 1**). Cancer was present in all but one of the patients with multiple antibodies, while cancer was detected in only seven of the 13 patients (54%) with only CRMP5 antibodies. Peripheral neuropathy (4 patients) and cerebellar ataxia (3 patients) were the most common PNS seen in patients with only CRMP5 antibodies and the patient with myasthenia gravis and thymoma was only positive for CRMP5.

The level of intensity of the line assays was evaluated by two independent researchers and varied from weak (+) to high intensity (+ + +). Five patients were graded as low intensity, 11 patients as medium intensity and eight patients were rated as high intensity by Ravo line assay. Four of these samples were negative by Euroimmun line assay, two of which were scored as low intensity by Ravo line assay, and two as medium intensity. Apart from these, the correlation in intensities between the two line assays was good (**Table 1**).

Detection of CRMP5-positive oligodendrocytes in cerebellar sections can be difficult to identify. If patients sera contain multiple antibodies, the CRMP5 staining can also easily be masked by other antibody staining. To increase the specificity of the oligodendrocyte staining, we used a commercial rabbit anti-CRMP5 antibody for co-staining (**Figure 1**). No CRMP5 staining was observed in 10 of the patient sera, while positive staining was observed in 14 patients (**Table 1**).

Twenty-one of the 24 patient sera were positive by CRMP5-CBA with a titer ranging from 1/10 to 1/100,000. None of the sera/CSF stained untransfected HEK293 cells, and no background staining was found in the 25 CRMP5 negative sera. No tumor was detected in the patients with a titer below 1/100. Seven of the sera that were positive by CRMP5-CBA were not positive in cerebellar sections, and all but one of these had an associated cancer. **Figure 1** shows a comparison of CRMP5 staining patterns in cerebellar sections and CRMP5-CBA. CRMP5-CBA identified CRMP5 antibodies in a patient that also showed clear CRMP5 reactivity in cerebellar sections (**Figure 1A**), in a patient with multiple antibodies where the CRMP5 signal was masked by additional staining of other antibodies (**Figure 1B**), and in a patient that were negative on cerebellar sections, but clearly positive by CRMP5-CBA (**Figure 1C**).

DISCUSSION

We evaluated commonly used assays for detection of CRMP5 antibodies in sera and CSF from 24 patients that were

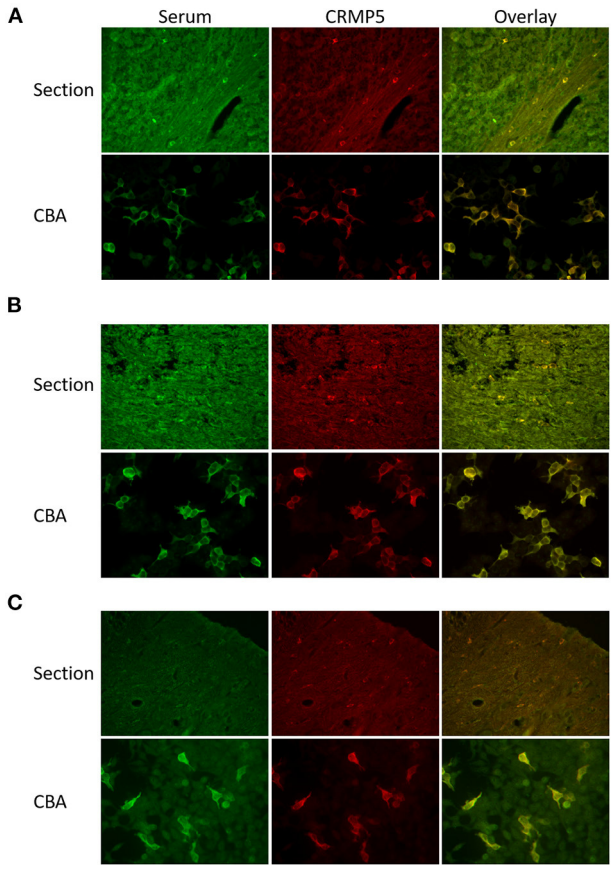

FIGURE 1 | Comparison of CRMP5 staining patterns in cerebellar sections and CRMP5-CBA. **(A)** Section: Serum from a patient with thymoma and myasthenia gravis stains oligodendrocytes in cerebellar white matter (green). A rabbit anti-CRMP5 antibody (red) stains the same cells. Overlay seen in yellow. CBA: Patient serum (green) specifically detect CRMP5-transfekted HEK293 cells. Anti-CRMP5 antibody (red) is used to detect CRMP5 positive cells. Overlay seen in yellow. **(B)** Section: Serum from a patient with peripheral neuropathy, lung cancer, Hu, Zic4, and CRMP5 antibodies. The CRMP5 signal is masked in the additional staining of the other antibodies in the patient serum (green). Co-staining with rabbit anti-CRMP5 antibody (red). The overlay shows that the serum also detects CRMP5 positive oligodendrocytes. CBA: The serum (green) stains CRMP5 positive cells in the CRMP5-CBA. Rabbit anti-CRMP5 (red) is used to verify CRMP5 positive cells. Overlay seen in yellow. **(C)** Serum from a patient with lung cancer and peripheral neuropathy (green) is negative in cerebellar sections.

identified as positive for CRMP5 antibodies by the Ravo line assay. We compared these results with those obtained using the Euroimmune line assay, immunofluorescence of cerebellar sections and a newly developed cell based assay for detection of CRMP5 antibodies (CRMP5-CBA), as well as with clinical data. There are also other tests commonly used for detecting paraneoplastic antibodies, like assays from Athena Diagnostics or assays used by the Mayo Clinic Laboratories, but there are to our knowledge no studies comparing such assays with the assays from Ravo or Euroimmun.

An associated tumor was found in 71% of the patients, with lung cancer being most prevalent. This in accordance with previous studies that have found that lung cancer and thymoma are most often associated with CRMP5 antibodies (3, 5, 7, 8). Further, we found that peripheral neuropathy was the most often reported PNS associated with anti-CRMP5 followed by cerebellar ataxia, which is also in accordance with previous reports (4, 6). We found additional paraneoplastic antibodies in 10 of the sera and showed that Hu and Sox1 antibodies were those most often identified. In two cases, both Hu and SOX1 antibodies appeared together with CRMP5, which is also in line with previous studies (3, 11). Identification of other autoantibodies co-expressed with CRMP5 is important as peripheral neuropathy has also been associated with Hu and Sox1 antibodies (12–14) and encephalopathy with Ri antibodies (15).

The Euroimmun line assay did not detect four of the 24 positive sera identified by the Ravo line assay, otherwise there was a good correlation between the two assays. This discrepancy might be explained by differences in how the recombinant proteins are produced. While Ravo uses a Baculovirus expression system for expressing full length CRMP5, Euroimmun expresses their proteins in a bacterial expression system.

Most laboratories use commercial line assays and /or immunohistochemistry to detect CRMP5 antibodies. While line assays are easy to perform, they can give false positive results (9, 10, 16). Therefore, another confirmatory test is needed for the line assays. Immunohistochemistry on cerebellar/brain stem tissue has so far been the preferred verification method for anti-CRMP5. However, this technique requires that the tissue is fixed in a specific, time-consuming way (1) which makes it not readily available in many laboratories. Further, even when the tissue is correctly fixed, identification of the CRMP5 positive oligodendrocytes can be difficult, as we show in our study. CRMP5 is expressed in a subpopulation of oligodendrocytes that are scarce in the white matter and in the brain stem. Hence, positive staining can easily be missed, especially if this staining is masked by staining of additional antibodies. In our study, we could detect positive staining in cerebellar sections only in 14 of the 24 sera/CSF, which suggests a significant under-diagnosis of CRMP5 positivity.

In view of the laborious nature of immunohistochemically analysis, we have substituted this technique with a newly developed assay, CRMP5-CBA. The CRMP5-CBA was positive for 21 of the 24 sera that were CRMP5 positive by the Ravo line assay. One of the 21 patient had a titer of 1/10 in the CRMP5-CBA. This is below serum cut-off defined by Euroimmun. However, we only had CSF from this patient and therefore interpreted the result as positive, as it is likely that the CRMP5 antibody level would be higher in a corresponding serum. Since the clinical diagnosis of this patient is brain infarct, it can be assumed that this is a false positive test even though loss of CRMP5 has been associated with brain ischemia in mice (17).

The rate of antibody detection has been shown to increase when both serum and CSF is tested (18). We only had complementing serum and CSF samples for four patients. In all cases, both serum and CSF were positive for CRMP5 antibodies (data not shown). Since the CRMP5-CBA has been optimized for serum testing, we chose to use the serum results in the cases where both serum and CSF were available.

No tumor was detected in seven of the 24 patients. Five of these had a follow up of more than 2 years, while two of the patients had a follow-up of 1 year. We cannot rule out that these two patients could develop cancer at a later time point. All cancer negative patients were CRMP5 antibody positive by the Ravo line assay and four were positive by the Euroimmun line assay whereas three were negative by CRMP5-CBA. Two of these cancer negative patients were strongly positive by CRMP5-CBA and IHC, and one patient had corresponding Hu antibodies by the two line assays. However, no cancer was found by PET scan in this patient (follow up of more than 10 years). Therefore, CRMP5 antibodies are not always correlated with a detectable tumor. Both patients had peripheral neuropathy or cerebellar ataxia, which are most often associated with anti-CRMP5. The other cancer negative patients had diagnoses that are not commonly associated with these antibodies (4). We do not have data on the rate of false-positives for CRMP5-CBA and IHC, but our data shows a good correlation between positive findings and clinical symptoms. Our CRMP5-negative control samples did not stain the CRMP5-CBA. To verify the rate of false-positives a larger control material is needed.

An in-house CRMP5-CBA assay has also been reported previously (19). Using this assay, the authors found that that four of 53 (7.5%) sera being positive by immune-histochemistry and negative by commercial line assays, were positive using their in-house CRMP5-CBA. Whether CRMP5-CBA is more sensitive than the commercial line assays is yet unclear. In our hands, screening sera/CSF for paraneoplastic antibodies by commercial line assays still requires confirmation by another immune assay. For CRMP5 detection, we found that the commercial CBA was more sensitive than immunohistochemistry and we therefore consider it a valuable add-on for verification of CRMP5 positivity in diagnosis of PNS.

DATA AVAILABILITY STATEMENT

The original contributions presented in 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 Regional ethics committee, Bergen, Norway (#242339). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CT was involved in design of the study, evaluated results, and wrote the manuscript. MH was involved in the design of the study, collected all the data, evaluated the results, and revised the manuscript. CV was involved in the design of the study, evaluated results, and was involved in writing the manuscript. All authors have approved the final version of the manuscript.

FUNDING

This study was funded by Helse Vest, project # F-12187.

ACKNOWLEDGMENTS

We thank Euroimmun for donating some of the CRMP5-CBA kits.

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frontiers in Neurology

REVIEW
published: 01 October 2021
doi: 10.3389/fneur.2021.706169

Paraneoplastic Neuropathies: What's New Since the 2004 Recommended Diagnostic Criteria

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The diagnostic criteria published by the PNS (Paraneoplastic Neurological Syndromes) Euronetwork in 2004 provided a useful classification of PNS, including paraneoplastic neuropathies. Subacute sensory neuronopathy (SSN) was the most frequently observed peripheral PNS, whereas other forms of neuropathy, as sensory polyneuropathy, sensorimotor polyneuropathy, demyelinating neuropathies, autonomic neuropathies, and focal nerve or plexus lesions, were less frequent. At the time of publication, the main focus was on onconeural antibodies, but knowledge regarding the mechanisms has since expanded. The antibodies associated with PNS are commonly classified as onconeural (intracellular) and neuronal surface antibodies (NSAbs). Since 2004, the number of antibodies and the associated tumors has increased. Knowledge has grown on the mechanisms underlying the neuropathies observed in lymphoma, paraproteinemia, and multiple myeloma. Moreover, other unrevealed mechanisms underpin sensorimotor neuropathies and late-stage neuropathies, where patients in advanced stages of cancer—often associated with weight loss—experience some mild sensorimotor neuropathy, without concomitant use of neurotoxic drugs. The spectrum of paraneoplastic neuropathies has increased to encompass motor neuropathies, small fiber neuropathies, and autonomic and nerve hyperexcitability syndromes. In addition, also focal neuropathies, as cranial nerves, plexopathies, and mononeuropathies, are considered in some cases to be of paraneoplastic origin. A key differential diagnosis for paraneoplastic neuropathy, during the course of cancer disease (the rare occurrence of a PNS), is chemotherapy-induced peripheral neuropathy (CIPN). Today, novel complications that also involve the peripheral nervous system are emerging from novel anti-cancer therapies, as targeted and immune checkpoint inhibitor (ICH) treatment. Therapeutic options are categorized into causal and symptomatic. Causal treatments anecdotally mention tumor removal. Immunomodulation is sometimes performed for immune-mediated conditions but is still far from constituting evidence. Symptomatic treatment must always be considered, consisting of both drug therapy (e.g., pain) and attempts to treat disability and neuropathic pain.

Keywords: cancer, tumor type, paraneoplastic neuropathy, onconeural antibodies, surface antibodies, mechanisms, therapy

OPEN ACCESS

Edited by:
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University of Utah, United States

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

Received: *06 May 2021*
Accepted: *30 August 2021*
Published: *01 October 2021*

Citation:
Zoccarato M, Grisold W, Grisold A, Poretto V, Boso F and Giometto B (2021) Paraneoplastic Neuropathies: What's New Since the 2004 Recommended Diagnostic Criteria. Front. Neurol. 12:706169. doi: 10.3389/fneur.2021.706169

INTRODUCTION

The paper published in 2004 by Graus et al. (1) on behalf of the PNS (Paraneoplastic Neurological Syndromes) Euronetwork provided a useful classification of PNS, including neuropathies. At that time, subacute sensory neuronopathy (SSN) was the most frequently observed neuropathy ("classical" paraneoplastic neuropathy) associated with cancer, while other entities, as sensory polyneuropathy, sensorimotor polyneuropathy, and demyelinating neuropathies, were less frequent. The other classical peripheral syndrome defined in 2004 was chronic gastrointestinal pseudo-obstruction. Several subsequent works have reported novel antibody associations, further types of tumor associations, and different types of neuropathy, several of which were not contained in the former classification. A recent update of the classification (2) confirmed the range of clinical presentations of neurological syndromes typically associated with cancer ("high-risk neurologic phenotypes"), including SSN. In this review we aim to provide an update on mechanisms, antibodies, clinical presentation, and management of paraneoplastic neuropathies focusing on the pathological entities.

MECHANISMS

The mechanisms underlying paraneoplastic neuropathies (PN) are manifold and not uniform for individual neuropathies or tumor types. Although paraneoplastic neuropathies have been known for a long time, the autoimmune hypothesis only appeared in 1965 when Wilkinson and Zeromski described antibodies against neurons in paraneoplastic sensory neuropathy for the first time (3). At the core of the autoimmune hypothesis is the production of antibodies against neural antigens determined by an immune response to cancer.

The main focus of the 2004 classification was on onconeural antibodies, targeting intracellular antigens shared by neuronal and tumoral tissues. The pathogenic role of onconeural antibodies remains unresolved. The most widely recognized hypothesis is that T-cell cytotoxicity accounts for neuronal cell loss in these conditions (4). Today, however, the mechanisms causing paraneoplastic neuropathies by far exceed the spectrum of onconeural antibodies (**Table 1**). Some peripheral conditions, e.g., peripheral nerve hyperexcitability syndromes (5), are mediated by neuronal surface antibodies (NSAbs). The term "surface antibodies" indicates antibodies targeting antigens present on the membrane of neurons, thus producing a potential direct effect, such as ion channel dysfunction.

Immune-mediated mechanisms determine also the rare presentations of Guillain-Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP), and vasculitis. Reference is occasionally made to these large groups of diseases in a paraneoplastic context. The exact trigger and relationship between the immune response and cancer is not clear, but it is doubtful that the same mechanisms apply to all these entities.

Plasma cell dyscrasia and other hematological entities present with a spectrum of different neuropathies of both axonal and demyelinating forms, like anti-myelin-associated glycoprotein (MAG) neuropathy, POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal-protein and skin changes) syndrome, and immunoglobulin light-chain (AL) amyloidosis. MAG and other antibodies targeting myelin-associated glycoprotein and glycolipids are deemed to be pathogenic (6). Hyperproduction of light chains could have a direct toxic effect but overall leads to the formation of amyloid deposits with extracellular accumulation of fibrils and consequent axonal damage. In addition, hyperviscosity effects have been claimed in Bing-Neel syndrome with peripheral involvement in Waldendström's macroglobulinemia (7).

A number of causes for paraneoplastic sensorimotor neuropathies, especially in advanced stages of cancer, remain obscure and resemble the development of neuropathies in some general diseases, as infections and critically ill conditions (See the paragraph on "Sensory neuromyopathy and terminal neuropathy").

By enhancing antitumor immunity, the emerging immune therapies, particularly immune checkpoint inhibitors (ICI), have been associated with a spectrum of immune-mediated diseases, including neuropathies and rapidly progressive polyradiculoneuropathies (8, 9).

TABLE 1 | Level of characterization and frequency of mechanisms underlying paraneoplastic neuropathies.

Associations/ mechanisms	Level of characterization	Frequency in the paraneoplastic neuropathy spectrum
Onconeural abs	+++	+++
NSAbs	+++	+
Other immune-mediated mechanisms	–	–
Hematologic diseases, including amyloid	++	++
Weight loss, cachexia, infection	–	+++

NSAbs, Neuronal Surface Antibodies.

ANTIBODY AND TUMOR ASSOCIATION

Several antibodies targeting neural antigens have been described in patients with PN, although this condition is well-known to occur also without antibodies (1, 10). The 2004 diagnostic criteria established that onconeural antibodies were consistently associated with PNS. Still they are now a fundamental marker supporting diagnosis of paraneoplastic neuropathy, commonly detected using commercial and in-house tests on cerebellar tissue with specific patterns and recombinant protein-based dot/line blotting (11). In 2004, onconeural antibodies were classified as "well-characterized" or "partially characterized" antibodies. This distinction was based on clinical relevance, number of published cases, a recognizable pattern on immunohistochemistry,

TABLE 2 | Onconeural antibodies reported in 2004 classification and associated with paraneoplastic neuropathy.

Antibody (antigen)	Types of neuropathy	Associated tumors	Selected published series after 2004 criteria
Hu (ANNA1)	SSN; autonomic neuropathy	SCLC	(12, 13, 17–19)
CV2 (CRMP5)	Sensorimotor neuropathy	SCLC; thymoma	(12, 13)
Amphiphysin	Polyradiculoneuropathy, SSN	Breast, SCLC	(14, 15)
ANNA3	Sensorimotor neuropathy	SCLC	/
PCA-2/MAP1B	Sensorimotor neuropathy; autonomic neuropathy	SCLC	(16)

availability of immunoblotting confirmation, and absence/low frequency in patients without cancer. The 2021 update of diagnostic criteria (2) classifies the antibodies associated with paraneoplastic diseases in three groups according to the frequency of association with cancer (high- >70%, intermediate-, and low- <30% risk antibodies), but in this review, we use the former nomenclature.

Well-characterized antibodies associated with paraneoplastic neuropathies included anti-Hu (ANNA-1), anti-CV2 (CRMP5), and anti-amphiphysin antibodies. Since 2004 the literature has confirmed that these three markers are consistently associated with the PN spectrum (12–19) (**Table 2**). SSN with anti-Hu antibodies is considered the most frequent PN (10). It is often the predominant manifestation of anti-Hu multifocal encephalomyelitis (paraneoplastic encephalomyelitis), which in 10% cases can also manifest with dysautonomic symptoms, as hypotension, dysrhythmia, or intestinal pseudo-obstruction (20). Two extensive case series have compared anti-Hu with anti-CV2 neuropathy (12, 13). Anti-CV2 neuropathy seems to be characterized more frequently by sensorimotor involvement and by asymmetric polyradicular involvement. SCLC has been confirmed as the most frequent cancer associated with both anti-Hu and anti-CV2 antibodies. Anti-amphiphysin antibodies are rare and associated with a broad spectrum of neurological manifestations, especially in women with breast cancer or SCLC. Peripheral involvement includes sensory neuronopathy, sensorimotor neuropathy, polyradiculopathy, and neuromyotonia (14, 15).

Partially characterized antibodies in 2004 included ANNA-3, a very rare reactivity reported in some cases of sensory, sensorimotor and autonomic neuropathy (21), anti-Zic4—a marker of cerebellar degeneration described in some cases of neuropathy, usually with concomitant antibodies (anti-Hu and/or anti-CV2) (22)—and anti-PCA2, which in the earlier large case series (23) was described in both central and peripheral syndromes, including LEMS and neuropathy. The real target of PCA-2 was identified in 2017, consisting of a microtubule-associated protein (MAP1B). PN is the most frequent presentation and was reported in about a half of anti-MAP1B patients with SCLC (16).

In the last 15 years, several novel onconeural antibodies related to PN have been reported. Most have been described in individual works, have not yet been confirmed by other research groups, and have a limited number of patients. The strength of these associations thus needs further characterization and confirmation before being considered relevant in the diagnostic approach to PN (**Table 3**).

In 2016, antibodies against 1,4,5-trisphosphate receptor type 1 (ITPR1) were characterized in patients with autoimmune cerebellar ataxia; this antibody was also found in three patients with sensorimotor polyneuropathy, associated in two cases with malignancy (adenocarcinoma of the lung and multiple myeloma) (24).

Antibodies targeting neurofilament light chain (NfL) have been reported as markers of ataxia and encephalopathy accompanying various cancers, especially neuroendocrine lineage neoplasms (25). Six out of 21 of the reported patients manifested with peripheral or cranial neuropathy.

Anti-KLHL11 is a recently described antibody identified in patients with brainstem encephalitis or cerebellar symptoms related to testicular and ovarian cancer (26, 27). A recent retrospective study of 32 patients manifesting a distinctive pattern of subacute paraneoplastic myeloneuropathy, associated with several neoplasms, identified the presence of anti-amphiphysin (8), anti-Hu (5), anti-CV2 (6), anti-Yo (1), anti-PCA2 (2), and one case of anti-KLHL11, or combinations of these (28).

Very recently, a further antibody reaction against Leucine Zipper 4 (LZUP4) was described in 28 patients with germ cell tumors (e.g., seminoma); four patients were affected with motor neuronopathy and polyradiculopathy (29).

In this context, the characterization of antibodies against Sry-like high mobility group box 1 (SOX1) deserves mention. In 2005, anti-glial nuclear antibody (AGNA) was identified as a marker of PNS-related lung cancer. The most frequent clinical association was LEMS, but SSN and sensorimotor neuropathy were also reported (30, 31). SOX1 was identified as the antigen in 2008 (32). As anti-SOX1 antibody is not a rare finding in patients with SCLC without paraneoplastic accompaniments (33), it can more properly be considered a serological marker of SCLC. Recently, the presence of SOX-1 was also advocated in non-paraneoplastic neuropathies (34, 35), but this finding was not confirmed in a later work (36).

In recent years several antibodies directed against NsAbs have been described, greatly expanding the interest in autoimmune neurological diseases. Antibodies against voltage-gated potassium channels (VGKC) were first discovered in

TABLE 3 | Novel antibodies associated with paraneoplastic neuropathy.

Antibody (antigen)	Localization (intracellular/surface)	Types of neuropathy	Associated tumors
AGNA/SOX1	Intracellular	Sensory neuronopathy, sensorimotor neuropathy	SCLC
ITPR1	Intracellular	Sensorimotor polyneuropathy	Lung, Multiple Myeloma
KLHL 11	Intracellular	Myeloneuropathy	Testicular seminoma
LZUP4 (Leucin-Zipper 4)	Intracellular	Motor neuropathy, polyradiculopathy	Germ cell tumors
NfL (Neurofilament light chain)	Intracellular	Peripheral and cranial neuropathy	Neuroendocrine lineage neoplasms
CASPR2	Surface	Neuromyotonia, painful neuropathy, Morvan syndrome	Thymoma (20–30%)
LGI1	Surface	Neuromyotonia, painful neuropathy	Thymoma (5–10%)
Netrin 1 receptors	Surface	Neuromyotonia	Thymoma
		Morvan syndrome	

the 1990s in patients with motor nerve hyperexcitability (neuromyotonia) and hyperhidrosis (37, 38). Anti-VGKC antibodies were later described in limbic encephalitis, with most cases being of non-paraneoplastic origin (39, 40). In 2010, it was established that anti-VGKC antibodies actually target two different associated proteins associated with ion channels, namely, LGI1 and CASPR2 (41, 42). These antibodies determine a continuous disease spectrum, often dominated by central nervous system involvement (limbic encephalitis) but frequently with relevant peripheral manifestations, including neuromyotonia, dysautonomia, and pain (43). Peripheral involvement, especially when isolated, is more frequent in CASPR2 than in LGI1 patients; this is probably due to the higher expression of CASPR2 in the peripheral nervous system, in the juxta-paranodal region (44). Morvan syndrome is another clinical picture associated with CASPR2 antibodies, in which peripheral hyperexcitability and dysautonomia coexist with psychiatric disturbances and sleep dysfunction. Recently, painful manifestations of LGI1 and CASPR2 autoimmunity have been highlighted. Sometimes pain is the cardinal symptom, especially in CASPR2 patients (45–47). As regards the associated tumors, anti-LGI1 diseases are rarely paraneoplastic, whereas a tumor, mostly a thymoma, can be detected in 20–30% of patients with CASPR2 autoimmunity. In patients with double positivity (anti-LGI1/anti-CASPR2), the likelihood of cancer is higher (up to 44%) (47).

Finally, a further surface reaction against Netrin-1 receptor has recently been described in patients with thymoma affected by Morvan syndrome or neuromyotonia and myasthenia gravis; the clinical relevance of this reactivity, which can be found together with CASPR2, needs further specification (48, 49).

Tumor association reflects antibody association (see **Tables 2**, **3**). The most frequent malignancy associated with PN is SCLC. However, other lung cancers, like adenocarcinoma, are not rare. Other associated malignancies are breast and ovarian cancer, and thymoma. Prostate cancer is an infrequent finding in the neurological paraneoplastic context, considering the frequency of the neoplasm, although cases of SSN have been reported with Hu antibodies (50). When the diagnosis of PNS is performed in a patient with an unknown or occult malignancy, oncological screening must be performed as recommended (51).

TYPES OF NEUROPATHIES

Over the decades, numerous individual observations have been published. Gradually, a core of generalized neuropathies has emerged, in particular, SSN. Several rarer types need to be considered, as do focal peripheral nerve lesions. The spectrum is probably still incomplete but reflects the present level of knowledge and experience (**Table 4**).

Generalized Neuropathies

Subacute Sensory Neuronopathy

SSN is the prototype of PN. It is characterized by asymmetry, acute/subacute onset in the upper extremities, and pain. The main clinical feature is sensory ataxia. Even though the motor system is not impaired in terms of strength, sensory loss results in ataxia and severe disability. The disease quickly progresses to a plateau phase, and has little or no tendency to improve. Electrophysiologic study reveals absent sensory responses; motor responses can be minimally altered (52). The neuropathology consists of inflammation in the dorsal root ganglia (DRG) and is often associated with posterior column degeneration. The therapeutic approaches are manifold (53) and usually immune modulation is recommended, although no evidence-based recommendations exist. SSN is a disabling condition and persons affected remain completely dependent. SSN is not entirely specific and has been observed as an idiopathic occurrence or in other autoimmune conditions as Sjogren's syndrome. The DRG can be affected by toxicity, as through platinum compounds and pyridoxine overdosage, but the extent of the clinical symptoms is not the same as in Hu-associated paraneoplastic SSN. SSN has also been observed in association with other PNS as cerebellar degeneration, PEM, brainstem involvement, limbic encephalitis, Lambert–Eaton myasthenic syndrome, and motor neuropathy. Patients may have evidence of autonomic involvement. A rare combination with myositis has been described (54). For the clinician, the appearance of an

TABLE 4 | Classification of paraneoplastic neuropathies.

Types of PN neuropathies	Sub-classification	Comments
Generalized neuropathies	Subacute sensory neuronopathy	Highly indicative of a PN cause- although also observed in other conditions
	Sensory neuropathy	Unspecific
	Sensorimotor neuropathy	Unspecific
	Sensory neuromyopathy and terminal neuropathy	Historic terminology
	Motor neuropathy	Rare
	Multiplex neuropathy	Vasculitis is rare
	Myeloneuropathy	Possibly new entity
	Autonomic neuropathies	Incidence uncertain
Rarer and disputed entities	Small fiber neuropathy	Incidence unclear, except for hematologically-associated types
	Cryoglobulinemic neuropathy	
	Hyperexcitability syndromes	
	Paraproteinemia and AL amyloid	
Focal nerve lesions	Cranial nerves	Individual cases
	Nerve plexus	
	Mononeuropathies	

SSN constitutes a strong recommendation to search for cancer, particularly SCLC.

Sensory Neuropathy

A pure sensory neuropathy is less specific for a paraneoplastic disease, and opinions regarding paraneoplastic etiology are controversial. Patients present with sensory symptoms in the typical glove-stocking distribution, often with neuropathic pain and a variable degree of incoordination (55). Onset is usually more insidious, following the length-dependent distribution. The most likely causes of a pure sensory neuropathy are autoimmune diseases (e.g., Sjögren) and toxic and metabolic neuropathies. The distinction between SSN and sensory neuropathy can be difficult; even in the 2004 classification it was not always certain how precise the distinction could be. Subacute or acute onset may resemble SSN. An attempt to differentiate ataxic from more painful sensory neuropathies was made by Oki et al. (18) and may help in differentiation.

Sensorimotor Neuropathy

Sensorimotor neuropathy (SMN) is generally the most frequent yet enigmatic neuropathy in terms of specific characteristics and etiology. The term implies a combination of sensory and motor symptoms of varying degrees. No specific or characteristic clinical items have been defined. Patients with SMN usually do not have severe neurological impairment. SMN has been observed as a paraneoplastic condition, also in association with onconeural antibodies (55, 56). The differential diagnosis is wide and ranges from other causes as alcohol, diabetes, and chronic idiopathic axonal polyneuropathy, particularly in individuals aged over 55 years (57).

Yet individual cases with SMN presenting as the first sign of cancer have been described. Practically speaking, SMN is uncharacteristic and the suggestion is to first exclude other possibilities before embarking on an extensive tumor search. At a later stage of the cancer, chemotherapy-induced peripheral neuropathy (CIPN) is the most likely differential diagnosis. In plasma cell dyscrasia and associated hematological diseases, a spectrum of neuropathies has been described, including SMN (see below).

Sensory Neuromyopathy and Terminal Neuropathy

Paraneoplastic sensory neuromyopathy is usually a late effect of cancer on the peripheral nervous system. It presents as a symmetric sensorimotor neuropathy, is usually mild, and can be associated with type 2 fiber muscle atrophy. It is not specific for individual cancers, is often associated with weight loss, and can be a general sign of advanced cancer. The sensory loss affects all qualities. Muscle weakness occurs in proximal and distal muscles (with so-called "intermediate sparing"). It is slowly progressing. Concomitant factors as chemotherapy or diabetes need to be ruled out. There is no specific antibody association at present. The nomenclature is historic and was not contained in the 2004 classification. However, in clinical practice, this type of neuropathy can be observed in advanced cancer patients.

The term "terminal neuropathy" refers to mild sensorimotor neuropathies observed in progressive general diseases, in advanced stages, including cancer, with or without concomitant use of neurotoxic drugs. This type of neuropathy can be likened to a common occurrence in patients with severe infections, i.e., weight loss, and its origin is probably different. For example, sarcopenia and cachectic neuropathy have been described in association with diabetic neuropathy (58, 59).

Motor Neuropathy

Pure motor neuropathy is rare. The term "lower motor neuron disease" has been used to identify patients with subacute development of generalized flaccid paresis with sparing of long tracts and bulbar muscles (60). This entity has been described infrequently and is not well-characterized. Only a few cases were collected in the PNS Euronetwork database (61), corresponding to <1% of identified patients. The term "lower

motor neuropathy" has been also used in conjunction with plasma cell dyscrasia and myeloma (62, 63), in hematological malignancies, and as a sequelae of local RT.

Multiplex Neuropathy

Despite being described in individual cases, paraneoplastic mononeuropathy multiplex is a rarePNS (64, 65). Mononeuropathy multiplex is generally associated with vasculitis. Chronic dysimmune neuropathies, as multifocal motor neuropathy (MMN), have been reported as PN (66), while rare cases of asymmetric neuropathies have been observed in AL amyloidosis (67) and in association with Waldenstrom's disease (68). Recently observed complications of ICI therapies also include reports of vasculitis (69). Notably, also other tissues can be involved by vasculitis as a clinical sign, e.g., skin vasculitis (70) and digital ischemia (71).

Myeloneuropathy

The simultaneous involvement of the spinal cord and peripheral nerves is termed myeloneuropathy. The usual underlying etiologies include B12 or copper deficiency, inflammatory/infectious diseases, and toxic diseases. This is a fairly new term in conjunction with PNS, which is not contained in classical descriptions. A recent paper (28) has described a number of cases that could be the basis for further considering its inclusion in the PNS classification. The series consists of 34 patients with various antibodies and cancers, especially SCLC and breast adenocarcinoma. Clinically they had a subacute, asymmetric presentation with sensory (e.g., paresthesias and impaired proprioception) and motor signs, as asymmetric weakness, and long tract signs. Pain and bladder dysfunction were frequent clinical findings. The concomitant presence of hyporeflexia and hyperreflexia at different sites was found in 81% of patients. MRI imaging showed spinal hyperintensity, which in about a half of patients was longitudinally extended. One third of patients showed lumbar nerve-root enhancement. Previous descriptions of similar syndromes are available (72, 73).

Autonomic Neuropathy

Autonomic features can be associated with several types of neuropathy. Symptoms can result in orthostatic hypotension, urinary symptoms, sweating abnormalities, intestinal pseudo-obstruction, gastrointestinal dysmotility, and subacute dysautonomia. Dysautonomia is typical of AL amyloid neuropathy. It has likewise been observed in several paraneoplastic forms of PN (74) and small fiber neuropathy (75). Another important aspect is the association of autonomic symptoms with other PNS, in particular with LEMS, but also in conjunction with paraneoplastic hyperexcitability syndromes as Morvan syndrome. In addition to areflexia, stocking-like sensory loss, and areflexia, autonomic symptoms also appear. Autonomic ganglionopathies have been observed in conjunction with ICI therapy (76). Notably, autonomic disturbances are also well-described in other common causes of neuropathy, as diabetes or neuropathies associated with systemic autoimmune diseases.

Rarer and Disputed Entities

Small Fiber Neuropathy

Small fiber sensory neuropathy (SFN) has not been part of the classical paraneoplastic spectrum. A recent study suggests that paraneoplastic causes can amount to 3% of SFN in association with onconeural antibodies that were not further specified (77). SFN has been documented in cases of Morvan syndrome (78). Although examples of SF involvement have been described in PN in solid cancers (79) and hematological diseases (80), it remains unclear whether the SF involvement is exclusive, or part of a general neuropathy syndrome.

Immune-Mediated Neuropathies

From the large, growing number of immune-mediated neuropathies, GBS, CIDP, and also rarely MMN have been mentioned to appear as a paraneoplastic phenomenon. In the PNS Euronetwork database, the incidence of this conjunction was low. The occurrence of both GBS and CIDP seems higher in hematological conditions (81, 82) and has also been described to be associated with other cancer types (74, 83) and with axonal loss. The occurrence of MMN as a PNS has been reported but seems to be extremely rare (84, 85).

Practically speaking, the appearance of either GBS or CIDP does not necessarily point to an underlying malignancy. Conversely, GBS may occur during the course of the disease in cancer patients with solid tumors (86) but can be the presenting phenotype in lymphoma or leukemia, and may be called "paraneoplastic" in these circumstances.

As a new development and based on immune mechanisms, several immune-mediated neuropathies, also resembling GBS, have been described to occur in conjunction with immune therapies, in particular with ICIs (9, 87).

Cryoglobulinemia

Cryoglobulinemia can occur as part of lymphoproliferative disease as in MGUS, macroglobulinemia, multiple myeloma (MM), leukemia, CLL, and immunoblastic lymphadenopathy. In addition to acrocyanosis, digital necrosis and purpura may occur. Likewise, signs of hyperviscosity, as thrombosis, can be associated. Cryoglobulins are monoclonal IgG, often IgM, and rarely light chain. Cryoglobulinemic neuropathy has been described as paraneoplastic, with IgM precipitation (88) and other conditions. Except for lymphoproliferative diseases, where the coexistence has been noted, it does not seem to be a frequent association.

Hyperexcitability Syndromes

Abnormal muscular activity with cramps, twitching and stiffness characterizes neuromyotonia and hyperexcitability syndromes. Electromyographic findings are typical (89). Acquired forms are considered immune mediated and can be found with underlying neoplasms, including in seronegative cases. Neuromyotonia can also present with symptoms and signs of sensorimotor and motor neuropathy, with no clear mechanisms (90). Neuropathic pain is a frequent finding in Morvan syndrome (see above) (91).

Plasma Cell Dyscrasia and Paraproteinemia

Plasma cell dyscrasias and paraproteinemias are usually not classified among paraneoplastic neuropathies. This is probably due to the inhomogeneous presentations within the different groups with paraproteinemia. Despite this, several types of neuropathies occur in association with the hematological disease by indirect involvement (not directly neoplastic), resembling the pattern of paraneoplastic disease. At least three conditions, such as POEMS syndrome, anti-MAG neuropathy, and AL amyloidosis, have characteristics of paraneoplastic disorders. The mechanisms are diverse and range from immunoglobulin (IgG) deposition to axonal and demyelinating neuropathies, hyperviscosity issues, and AL amyloid deposition.

Neuropathies related to MGUS are usually sensory-motor, axonal, or demyelinating. Commonly, IgG or IgA cause axonal lesions, whereas IgM tends to be associated with demyelinating features.

Waldenstrom's disease can develop from MGUS and is considered as a low-grade lymphoma (lymphocytoplasmatic lymphoma). It can be associated with demyelinating neuropathies, sometimes with MAG antibodies. Hyperviscosity mechanisms can also result in a multifocal neuropathy (92). Cryoglobulinemia can be associated; AL amyloidosis is rare.

Multiple myeloma presents with a variety of PN, including axonal and demyelinating types with no typical clinical features. AL amyloidosis is responsible for 30–40% of neuropathies in myeloma, in particular with λ light chain.

Three phenotypes further illustrate the relationship between PNS and plasma cell dyscrasia. Anti-MAG neuropathy results in a painful sensory neuropathy, predominantly in the legs with impaired balance. Progression is slow and leads to sensory ataxia. Tremor action is frequently reported. Numerous investigations have discussed the effects of anti-MAG and the role of sulfoglucuronyl-glycosphingolipid (SGPG) antibodies (6). Therapy suggestions are controversial, among them anti-CD20 drugs, like rituximab, and attempt to neutralize the MAG antibodies (93).

POEMS syndrome is considered by many to be a PNS. The disease is often rapidly devastating, resulting in neuropathy with tetraparesis and multi-organ involvement. The neuropathy is M-protein related (Ig A or G) and can cause axonal, demyelinating, or mixed neuropathy (61). Often, an isolated osteosclerotic lesion can be detected. The distinction between CIDP and POEMS can be difficult and neurophysiological criteria have been suggested (94). A CIDP-like presentation with pain could be a useful indicator for POEMS. Elevated levels of vascular endothelial growth factor (VEGF) are found in about two-thirds of patients (95).

Light-chain (AL) amyloidosis is an important differential diagnosis in paraproteinemia, Waldenstrom's disease, multiple myeloma, and several other entities. Clinically, a combination of fatigue, renal impairment, sensory and autonomic neuropathy, and often also carpal tunnel syndrome (CTS) is suggestive. In addition to the characteristic sensory, often painful neuropathy, frequently including autonomic features, focal lesions due to deposits of amyloid are also described. Macroglossia, periorbital purpura, congestive heart failure, and orthostatic hypotension are other typical (but not specific) clinical findings (96) (**Table 5**).

TABLE 5 | Neuropathies associated with hematological diseases.

Hematological disease	Neurological syndrome
MGUS	Anti-MAG neuropathy (*), POEMS
Waldenstrom's disease	Axonal, demyelinating, anti-MAG neuropathy (*), hyperviscosity syndromes
Multiple myeloma	Axonal, demyelinating, AL amyloid (*)
Lymphoma	Immune-mediated (*), rarely AL
Leukemia	Rarely AL amyloid in CLL (*) and hairy cell leukemia

**Neuropathies which bear similarities with the characteristics of paraneoplastic syndromes.*

Focal Nerve Lesions

Focal paraneoplastic neurological syndromes are rare and usually not contained in the classifications. The list of entities provided here is incomplete and based on observations and case reports, lacking consistency and systematic approach. Focal presentations include cranial nerve lesions, plexopathies, mononeuropathies (e.g., CTS) (97), and also muscle involvement.

Cranial Neuropathies

There are numerous reports on paraneoplastic cranial nerve (CN) lesions (**Table 6**). Three CNs appear to be preferably affected: the optic, trigeminal, and vestibular nerves. In addition to lesions of the optic nerve (98, 99), also in combination with spinal cord lesions (100), there are several visual conditions, as cancer-associated retinopathy (101), melanoma-associated retinopathy (102), acquired night blindness, bilateral diffuse uveal melanotic proliferation, bilateral diffuse uveal melanotic proliferation, cone dystrophy and achromatopsia, and photophobia (103).

Oculomotor nerve lesions are rarely described as a PN. Local neoplastic causes and orbital myositis need to be excluded (104). However, extraocular muscle involvement has been described in AL amyloidosis, and local AL deposits in the lid may cause ptosis.

The trigeminal nerve can be affected in autoimmune and rheumatoid arthritis. Similarly, sensory trigeminal neuropathy, or the "numb chin" (105) and "numb cheek" (106) syndrome subtypes, have been reported to be caused by paraneoplastic mechanisms (107, 108). The presence of orofacial pain (109–111) and neuralgia have been suggested to be of paraneoplastic origin, among other causes. Vestibular damage and neuritis have been described as paraneoplastic phenomena in a few selected cases (112–114). There is a paucity of reports on possible damage to the caudal cranial nerves. Local amyloid depositions (115) in the soft palate and tongue (116) need to be considered under plasma cell dyscrasia. Several cases of possible multiple CN lesions, caused by a potential paraneoplastic mechanism, have been mentioned (117, 118). A similar distribution of affected CNs

TABLE 6 | Paraneoplastic involvement of cranial nerves.

CN	Neoplastic	Paraneoplastic syndromes	Other mechanisms
II	Base of the skull	Optic nerve neuropathy/neuritis	Other immune-mediated causes, hyper-viscosity
	Leptomeningeal carcinomatosis	CAR, MAR	
III, IV, VI	Base of the skull tumors/metastasis	Individual cases	/
	Orbital metastasis		
	Leptomeningeal carcinomatosis		
V	Local metastasis, base of the skull metastasis, mandibular metastasis	Numbness trigeminal nerve, or parts (chin, cheek)	Isolated facial pain, neuralgia
VIII	Leptomeningeal carcinomatosis	Few reports on vestibular neuritis	/
Caudal CNs	Base of the skull metastases	Not reported (isolated nerves)	Focal amyloid in plasma cell dyscrasia
	Leptomeningeal carcinomatosis		
	Lesions after the exit of CN of the bony skull		
Multiple CNs	Base of the skull metastasis	Few reports, not homogeneous	/
	Leptomeningeal carcinomatosis		

CAR, cancer associated retinopathy; MAR, melanoma associated neuropathy.

has been reported as a complication of treatment with immune checkpoint inhibitors (119) (see below).

Plexopathies

PN causing plexopathies are rare but have been reported in individual cases. Most reports date back some years, and new investigations, in particular with the aid of imaging techniques, could potentially detect a symptomatic cause. The cervical plexus is mentioned in regard to the phrenic nerve (120–122). Although there are reports that inflammatory paraneoplastic causes can affect the brachial and lumbosacral plexus, the evidence is based on individual case reports (123).

Mononeuropathies

In plasma cell dyscrasia, CTS could be a sign of amyloidosis. The issue of other isolated paraneoplastic mononeuropathies is uncertain. One report suggests a paraneoplastic ulnar nerve lesion (124); there is also mention of a peroneal nerve lesion (125). Multiple enlarged nerves (126) have been described in ultrasound as a PNS, but may lack a clinical correlate. Deposition of light chains causing individual nerve lesions has been described (97). In summary, the appearance of a mononeuropathy of paraneoplastic origin is not likely, except in the case of CTS associated with AL amyloid.

CANCER THERAPY IN DIFFERENTIAL DIAGNOSIS

CIPN is the commonest form of neurotoxicity in cancer patients, causing a chronic neuropathy in about 30% and milder/temporary symptoms in up to 70% of those undergoing traditional chemotherapy (127). In addition to the burden on patients' quality of life, CIPN can lead to modifications or discontinuation of oncologic therapy, thus impacting on their overall prognosis. Even after cessation of chemotherapy, symptoms impacting the quality of life persist in half of patients (128). Alteration of cancer treatment due to CIPN involves 10–65% of patients; in up to one third of patients, chemotherapy needs to be discontinued (129).

Despite significant variability in susceptibility [depending on concurrent diabetes, alcohol consumption, and other pre-existing neuropathies, along with genetic factors and older age at onset (130)], the development of CIPN is generally dose-dependent and thus influenced by different drug schedules and combinations, as well as route of administration. Traditionally, CIPN involves breast and colon cancer patients undergoing toxic doses of intravenous (IV) cisplatin and oxaliplatin. The cumulative toxicity mainly affects the DRG and their axons, causing a sensory neuronopathy or dying-back neuropathy; small fiber neuropathies can also ensue, while motor, autonomic, and cranial involvement is less common.

Most patients develop CIPN in the first three or four cycles of treatment, with gradual progression of symptoms and subsequent stabilization at or soon after treatment completion. Patients typically display length-dependent symmetric sensory loss (sometimes even leading to ataxia) with prominent acral sensory symptoms, pain episodes, and reduced dexterity. A notable exception is platinum-associated neuropathy, as oxaliplatin may also cause distinctive acute, transient, cold-induced dysesthesias after the first infusions, while both oxaliplatin and cisplatin neurotoxicity usually worsens in the months following the end of chemotherapy (i.e., the coasting phenomenon). CIPN then slowly improves with time, sometimes leaving a chronic pain syndrome with dysesthesia, hyperalgesia, tactile and thermal allodynia, and spontaneous pain. Acute pain syndrome related to nerve pathology has also been reported with paclitaxel (131). New anti-microtubule drugs can induce axonal, mainly sensory polyneuropathy, as can older taxanes [e.g., eribulin and ixabepilone (132)].

Recently, advancements in more selective targeted therapies and newer agents have improved chemotherapy tolerability and overall cancer survival but have not translated into a reduction of CIPN cases. Besides having to differentiate CIPN from other conditions that do not dictate premature treatment discontinuation, the advent of biological agents, known to have more idiosyncratic and off-target side-effects, has further added to the neurologist's diagnostic conundrum. Indeed, CIPN pathogenesis largely remains to be defined and is still one of the most common dose-limiting complications of antitumor treatment, considering the growing number of cancer survivors who develop late drug-resistant chronic pain syndromes that may heavily impact on their quality of life.

Targeted Therapies

Tumor-specific antibodies cause similar, unexpected nerve injury ranging from mild, predominantly sensory neuropathies (i.e., polatuzumab vedotin and enfortumab vedotin) to potentially severe motor neuropathy, as seen in lymphoma patients undergoing brentuzumab (133, 134); these generally improve slowly upon discontinuation. Neuropathies have also been reported in high numbers during adotrastuzumab emtansine treatment (135). Regarding the latest drugs, such as neurotrophic-tyrosine-receptor-kinase gene and anaplastic-lymphoma-kinase inhibitors, neuropathy has been described with both larotrectinib [including grade III and IV reactions within 3 months of treatment (136)] and lorlatinib (137), respectively.

Immune Checkpoint Inhibitors

Immune checkpoint inhibitors (ICIs) are monoclonal antibodies directed against specific molecules on activated immune cells whose role is to maintain self-tolerance and prevent autoimmunity, thus enhancing anti-tumor immunity. Targets include programmed death-1 receptor (nivolumab, pembrolizumab, cemiplimab), its ligand (atezolizumab, avelumab, durvalumab), and cytotoxic T lymphocyte antigen-4 (ipilimumab). Alongside, ICIs may cause off-target toxicities called immune-related adverse events (irAEs) (138, 139). Neurological irAEs represent a minor proportion, with an estimated incidence of 1–3%, more common after combination checkpoint blockade (140, 141). Nevertheless, they are clinically relevant complications with high-grade toxicity, carrying significant morbidity and mortality (142), and thus requiring prompt identification. Immune-related peripheral nerve disorders induced by ICI are highly heterogeneous (8, 9). Cranial neuropathies and polyradiculoneuropathies have emerged as the most common phenotypes (9). Cranial neuropathies may be isolated or associated with other neurological manifestations, frequently involving facial and abducens nerves, although any CN may be affected (143) Acute and chronic polyradiculoneuropathies mostly include GBS or CIDP and usually develop within the first three cycles of therapy (9, 144). Unlike classical GBS, cerebrospinal fluid analysis often reveals lymphocytic pleocytosis besides hyperproteinorrachia (142) and benefits from steroid administration. Other reported ICI-related neuropathies are mononeuritis multiplex/vasculitic neuropathy (8, 9), small fiber/autonomic neuropathy (145), sensory neuronopathies, and neuralgic amyotrophy (146, 147).

Despite these findings, ICIs are generally considered safer than conventional chemotherapy for the peripheral nerve, and ICI-induced neuropathies are more likely to have an acute/subacute and non-length-dependent presentation (9, 146).

According to current guidelines (148), management requires ICI discontinuation and high-dose IV steroid administration, followed by slow steroid tapering to avoid relapses related to the long-lasting half-life of ICIs (146). Up to 50% of cases (149) could be resistant to steroid therapy and, due to their severity, require IV immunoglobin (IG), plasma exchange, or immunosuppressants.

CAR T Cell Therapy

Chimeric antigen receptor T-cell therapies have become standard treatments of relapsed or refractory hematological malignancies. Acute neurotoxicity is well-documented and moderate-to-severe neurological events are estimated to occur in 20–30% of patients, with a median time of onset of 5–6 days after infusion (150–152). Neurological manifestations are associated with cytokine release syndrome, referred to as "immune effector cell-associated neurotoxicity syndrome," which identifies a distinctive encephalopathy manifesting with stereotypic evolution of a specific set of symptoms (152). Very little is known about potential long-term adverse events; however, a case of peripheral neuropathy has been described as a late complication (153).

THERAPY

The general therapeutic strategy for PNS is based on the assumption that the detection of cancer and its removal can improve the neurological syndrome. This is seldom obtained in PN associated with onconeural antibodies. Numerous attempts have been published for the treatment of SSN. A systematic review in 2012 concluded that only class IV evidence was available for the effect of IVIG, plasma-exchange, steroids, or immune-suppressive chemotherapy (53). Most individual recommendations suggest immune-modulating therapies can stop the progression of the PN. Given the severe debilitating outcomes in patients with SSN, these interventions are justified but should be performed very early (e.g., 2 months) after neurological symptoms onset (154). On the other hand, diseases associated with NSAbs are usually responsive to immunotherapy. This also seems to be confirmed for peripheral involvement in syndromes with anti-LGI1 anti-CASPR2 antibodies (47). Steroids are often the first option, followed by or associated with IVIG and plasma exchange. Therapies with anti-CD20 treatment (e.g., rituximab), or immunosuppressors like cyclophosphamide, have less supporting evidence than do other NSAbs associated diseases (e.g., NMDAR encephalitis) (155). Nevertheless, these options can be considered in non-responsive cases and their efficacy has been reported (44, 78, 156).

The smaller group of acute immune-mediated neuropathies, as GBS, CIDP, and vasculitis, usually respond to conventional immune therapies, approved for the non-neoplastic entity of the given neuropathy. Paraproteinemic neuropathies and AL

amyloidosis are also treatable to some extent, according to current hematological guidelines.

The appearance of PN has implications not only for cancer and cancer therapy but also significantly impacts patient performance, quality of life, and disability. Symptomatic therapy is therefore a cardinal feature of treatment. Pain control is a key goal. This can be achieved by antidepressants (tricyclic antidepressants and serotonin-noradrenaline reuptake inhibitors) and by GABA-mimetic drugs (first-line therapy). Second- and third-line drugs for neuropathic pain include topical lidocaine and opioids (157). Neuromyotonia can respond to antiepileptics as carbamazepine, phenytoin, lamotrigine, and sodium valproate (158).

Cancer rehabilitation is an important initiative to promote specific therapies for patients affected by neurological conditions in cancer. Therapy effects and, due to increased long-term survival, persisting effects need to be specifically treated. The rehabilitation of neuropathies is generally also heterogeneous, depending on the focus of deficit and disability. The reference level could be at best adapted and copied from the rehabilitation of cancer patients with CIPN, which bears similarities with the most common phenotype.

CONCLUSION

In summary, since the 2004 classification, several peripheral manifestations have been described, mainly with new antibodies, in patients affected by cancer. Whereas novel intracellular antibodies need more robust evidence to become relevant in clinical practice, the true novelty has been the discovery of NSAbs in diseases with prominent, or coexisting, peripheral involvement, which can be paraneoplastic.

Circulating autoantibodies are commonly considered a valuable tool for cancer diagnosis and are frequently requested in clinical practice for patients with unclear neurological peripheral symptoms. However, proper adherence to the use of biomarkers is critical in translating recommendations into clinical practice. At present, circulating classical onconeural antibodies and only a few NSAbs are considered a valuable tool for cancer diagnosis for patients with paraneoplastic neuropathy.

Finally, new cancer therapies seem to evoke immune-mediated neurological syndromes, which may mimic PN, but appear at different times during treatment. In the coming years, the study of the mechanisms and effects of these therapies will provide new insights into the relationship between cancer, immunity and the nervous system.

AUTHOR CONTRIBUTIONS

WG, MZ, AG, and BG conceived and drafted the review. VP and FB contributed to draft the review. WG and BG revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors thank Joanne Fleming for English editing.

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frontiers in Neurology

CASE REPORT
published: 22 October 2021
doi: 10.3389/fneur.2021.728700

Case Report: A False Negative Case of Anti-Yo Paraneoplastic Myelopathy

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OPEN ACCESS

Edited by:
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Reviewed by:
Tomoko Okamoto, National Center of Neurology and Psychiatry, Japan
Yujie Wang, University of Washington, United States
Sean J. Pittock, Mayo Clinic, United States

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

Received: *21 June 2021*
Accepted: *16 September 2021*
Published: *22 October 2021*

Citation:
Bartley CM, Parikshak NN, Ngo TT, Alexander JA, Zorn KC, Alvarenga BD, Kang MK, Pedriali M, Pleasure SJ and Wilson MR (2021) Case Report: A False Negative Case of Anti-Yo Paraneoplastic Myelopathy. Front. Neurol. 12:728700. doi: 10.3389/fneur.2021.728700

The development of autoimmune antibody panels has improved the diagnosis of paraneoplastic neurological disorders (PNDs) of the brain and spinal cord. Here, we present a case of a woman with a history of breast cancer who presented with a subacute sensory ataxia that progressed over 18 months. Her examination and diagnostic studies were consistent with a myelopathy. Metabolic, infectious, and autoimmune testing were non-diagnostic. However, she responded to empirical immunosuppression, prompting further workup for an autoimmune etiology. An unbiased autoantibody screen utilizing phage display immunoprecipitation sequencing (PhIP-Seq) identified antibodies to the anti-Yo antigens cerebellar degeneration related protein 2 like (CDR2L) and CDR2, which were subsequently validated by immunoblot and cell-based overexpression assays. Furthermore, CDR2L protein expression was restricted to HER2 expressing tumor cells in the patient's breast tissue. Recent evidence suggests that CDR2L is likely the primary antigen in anti-Yo paraneoplastic cerebellar degeneration, but anti-Yo myelopathy is poorly characterized. By immunostaining, we detected neuronal CDR2L protein expression in the murine and human spinal cord. This case demonstrates the diagnostic utility of unbiased assays in patients with suspected PNDs, supports prior observations that anti-Yo PND can be associated with isolated myelopathy, and implicates CDR2L as a potential antigen in the spinal cord.

Keywords: paraneoplastic neurologic disease, myelopathy, anti-Yo antibodies, phage display, breast cancer, CDR2L

INTRODUCTION

The initial evaluation of subacute or chronic progressive myelopathy includes investigations into structural, metabolic, inflammatory, and vascular causes. Inflammatory etiologies include infections due to organisms such as *Treponema pallidum*, varicella zoster virus, HIV-1, and *Borrelia burgdorferi* as well as autoimmune diseases like multiple sclerosis, neuromyelitis optica spectrum disorders, and paraneoplastic neurological disorders (PNDs). The diagnosis of a PND is supported by the detection of a recent or new malignancy and can be confirmed by the identification of

autoantibodies associated with a specific PND in the blood and/or cerebrospinal fluid (CSF) (1). Moreover, identification of a specific PND autoantibody can guide a targeted search for unidentified neoplasms.

As the number of diagnostic paraneoplastic autoantibodies has grown, panels have been developed that simultaneously test for multiple antibodies to facilitate clinical diagnosis and management. Testing is often performed in two stages, with initial screening by indirect immunofluorescence assay (IFA) and/or cell-based assays (CBAs) followed by confirmatory reflex testing (CBAs or immunoblotting) if the initial screen suggests the presence of a paraneoplastic antibody. Although PND panels are sensitive and specific, they test for a limited number of autoantibodies, and cases with a high degree of clinical concern for PND are often seronegative. To expand the ability to detect autoantibody targets in human disease, phage immunoprecipitation sequencing (PhIP-Seq) with a programmable peptide library representing the whole human peptidome was developed (2–5). PhIP-Seq has led to the identification of novel autoantibodies in undiagnosed PNDs, and high-resolution epitope mapping by deep mutational scanning with phage in previously characterized PNDs (2, 6, 7).

Here, we present a patient with sensory ataxia secondary to a myelopathy for whom clinical PND autoantibody screening was negative and therefore did not undergo reflex testing. Unexpectedly, research-based PhIP-Seq identified antibodies in the CSF against anti-Yo antigens cerebellar degeneration related protein 2 like (CDR2L) and cerebellar degeneration related protein 2 (CDR2) that were subsequently orthogonally validated.

CASE DESCRIPTION

A 36-year-old woman with a history of breast cancer presented to our neurology clinic. She was previously diagnosed with infiltrating carcinoma of the left breast. Pathology had demonstrated an aggressive neoplasm with high nuclear grade and extensive perineoplastic lymphocytic infiltration [**Figure 1A**, WHO 2019 (8) classification "Invasive carcinoma of no special type," T2N0, human epidermal growth factor receptor (HER2) positive, estrogen receptor, and progesterone receptor negative]. She was treated with pertuzumab, trastuzumab, and docetaxel for four cycles. Two months after her diagnosis and initiation of chemotherapy, she developed difficulty with her gait and sensory loss in her feet. Five months after her diagnosis, she underwent bilateral mastectomy, demonstrating no residual disease (**Figure 1B**). She subsequently completed a year of monthly trastuzumab.

Upon presentation to our clinic, 17 months after her diagnosis (15 months after the onset of her neurological symptoms), she noted difficulty maintaining balance when her eyes were closed and found it increasingly challenging to walk in a straight line. Her symptoms had progressed to the extent that she was now ambulating with the assistance of a cane. She endorsed mild sensory loss below her knees and denied any involvement of her upper extremities. She denied any vision changes, dysarthria, dysphagia, weakness, pain or bowel or bladder dysfunction.

Her general examination was unremarkable, and her neurological examination revealed no abnormalities with her mental status, cranial nerves, or strength. Sensory examination was notable for distal large fiber sensory loss, with reduced vibration below the knees with absent vibration in the feet and ankles and mild (10–20%) loss of light touch below her knees. Reflexes were present in the biceps and triceps but absent at her knees and ankles. The Romberg sign was present, and she had a wide-based, unsteady gait.

CLINICAL COURSE AND DIAGNOSTIC ASSESSMENT

Evaluations prior to her presentation to our clinic were notable for normal contrast-enhanced magnetic resonance imaging (MRI) of her brain, and normal electromyography and nerve conduction studies (EMG/NCS). She had repeat EMG/NCS studies at our institution that were also unremarkable and unremarkable serological studies including a vitamin B12 level of 694 ng/L (**Supplementary Table 1**). She had normal median nerve somatosensory evoked potentials (SSEPs) from Erb's point, C5 spine and the cortex but abnormal tibial nerve SSEPs. With stimulation of the left posterior tibial nerve, a normal popliteal fossa response and P37 response could be seen (normal amplitude, latency, and inter-peak latency). However, the lumbar potential was poorly formed. With stimulation of the right posterior tibial nerve, a popliteal fossa, lumbar potential, and P37 response could be seen, but the latency to peak of the P37 component as well as the inter-peak latency between the popliteal fossa, lumbar potential and the P37 were delayed.

Given her exam and electrodiagnostic study results, a localization to the lumbosacral sensory nerve roots was initially favored. To further investigate, she had a lumbar puncture and a contrast enhanced MRI of the lumbar spine. CSF studies were without evidence of inflammation or malignant cells (**Supplementary Table 1**), and imaging did not identify any abnormalities in the lumbar spine or nerve root enhancement as is typically seen in chronic immune sensory polyradiculopathy (CISP). Her chemotherapy exposure was also considered as a potential cause. However, pertuzumab does not have any established neurotoxic effects. Trastuzumab and docetaxel are known to cause a polyneuropathy, but there was no evidence for a polyneuropathy on exam or EMG/NCS (9).

Three months after her initial visit, she reported worsening symptoms. On exam, her loss of sensation to light touch had worsened below her knees (50–60% loss) and now extended to mild involvement of her thighs. Vibration was absent up to her hip, and her gait instability was more prominent. Given her worsening symptoms and history of malignancy, inflammatory conditions affecting the spinal cord and/or nerve roots were still part of the differential diagnosis despite a negative work-up thus far. She was started empirically on monthly intravenous immunoglobulin (IVIg, total dose of 2 g/kg divided over 3 days). After three doses of IVIg, she reported stabilization in her sensory loss and felt her gait had improved. On monthly IVIg, she reported improvement in sensation above her knees.

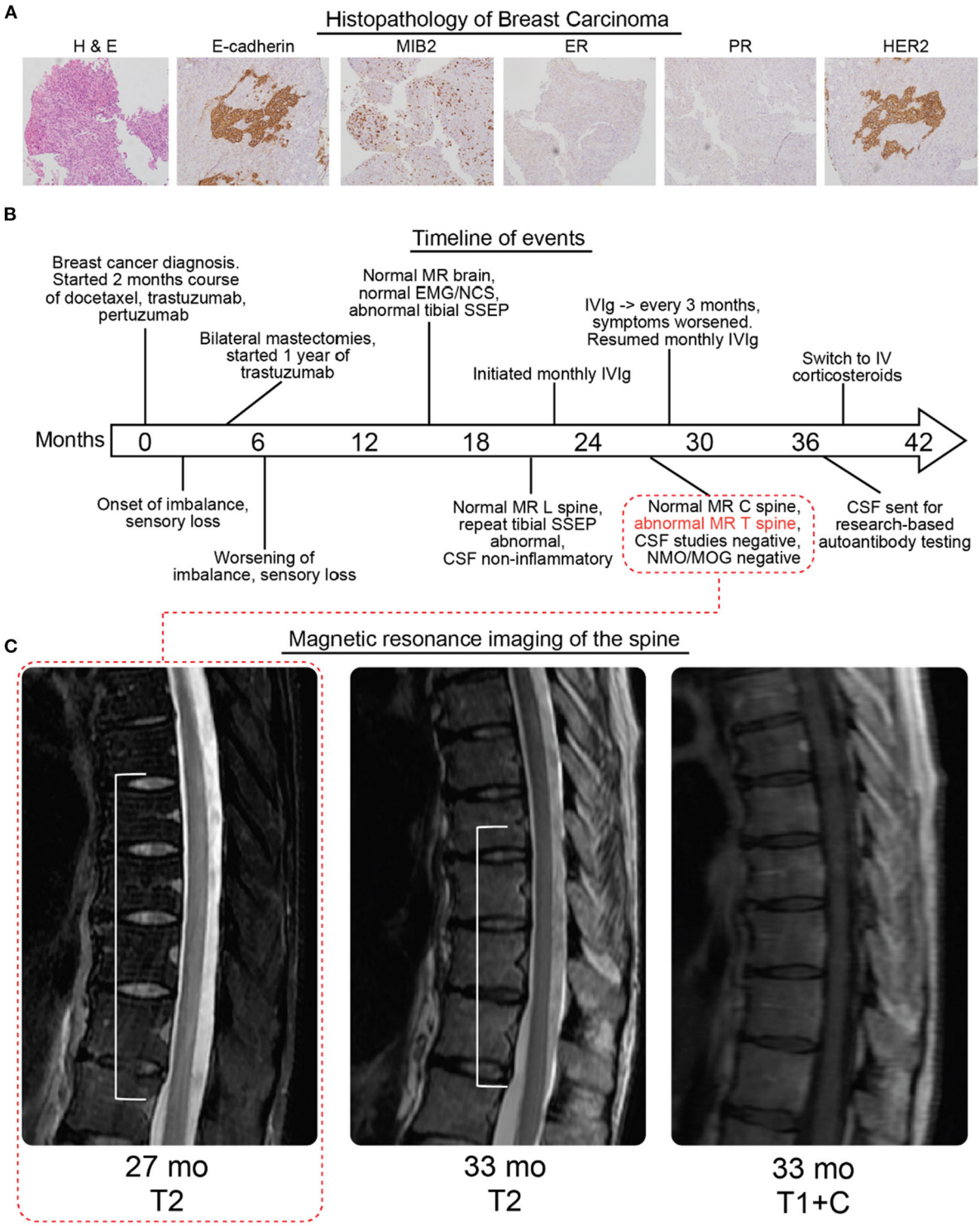

FIGURE 1 | Histopathology, timeline, and radiologic findings. **(A)** Hematoxylin and eosin staining (left panel) followed by molecular characterization of patient breast carcinoma (MIB2, mindbomb E3 ubiquitin protein ligase 2; ER, estrogen receptor; PR, progesterone receptor; HER2, Erb-b2 receptor tyrosine kinase 2). The carcinoma was rated as E-cadherein and MIB2 expressing, ER/PR negative, and HER2 positive. **(B)** Timeline of diagnosis, symptoms, imaging, electrodiagnostic studies, and treatment. Imaging corresponding to 27 weeks is indicated in the first subpanel one of **(C)**. **(C)** MRI of the spine without gadolinium at 27 months showed a dorsally predominant T2 hyperintense signal spanning T5–T10. At 33 months there was some improvement with the T2 hyperintense signal now spanning T7–T10 which was not contrast enhancing (additional views and brain MRI in **Supplementary Figure 1**). All times are relative to the initial diagnosis of cancer, neurological symptoms began 2 months after the cancer diagnosis.

When treatment frequency was reduced to every 3 months, her symptoms worsened, prompting return to the monthly IVIg (**Figure 1B**).

A serum autoimmune encephalopathy panel was sent prior to IVIg administration, including for PNDs associated with sensory neuropathies and neuronopathies (such as anti-CRMP5 and anti-ANNA1), and was unremarkable (Mayo Clinic, Rochester, MN, test ID: ENS2) (10). An antibody panel for autoimmune etiologies of neuromuscular syndromes was sent after two doses of IVIg and revealed low titers of IgG antibodies to beta-tubulin, high titers of IgM antibodies to tri-sulfated heparin disaccharide (TS-HDS), and low titers of IgG antibodies to fibroblast growth factor receptor 3 (FGFR3) (Washington University, St. Louis, MO) (**Supplementary Table 1**). This pattern of abnormalities has been associated with sensory axonal neuropathies, but it is not specific (11, 12).

Repeat imaging of the brain, and first-time MRI of the cervical and thoracic spine without gadolinium were performed 27 months after her cancer diagnosis (25 months after onset of neurological symptoms), revealing a longitudinally extensive, dorsally predominant T2 hyperintensity from T5 to T10 (**Figure 1C**, **Supplementary Figure 1**). Repeat imaging of the thoracic spine with contrast 33 months after her cancer diagnosis and after receiving IVIg for about 1 year demonstrated less conspicuous and less extensive T2 signal from T7 to T10 with no contrast enhancement.

Two additional CSF exams showed no pleocytosis, no oligoclonal bands, no malignant cells, and a normal IgG index. Testing for anti-AQP4 and anti-MOG antibodies in the serum was negative (**Supplementary Table 1**). CSF was sent for an autoimmune encephalopathy panel and was negative (Mayo Clinic, test ID ENC2).

IDENTIFICATION OF CANDIDATE ANTI-YO ANTIBODIES BY PHAGE IMMUNOPRECIPITATION SEQUENCING

Given the patient's apparent response to IVIg, history of breast cancer, and negative clinical autoantibody testing, we screened her CSF for novel candidate autoantibodies using PhIP-Seq. Our phage display library is comprised of 49 amino acid peptides with a 25 amino acid overlap that together encode all known and predicted human proteins and their isoforms (7). To pan for candidate autoantibodies by PhIP-Seq, CSF is incubated with the PhIP-Seq library, IgG binds to target peptides displayed by individual T7 bacteriophage, IgG is isolated using protein A/G magnetic beads, and IgG-bound phage are sequenced to determine which human peptide they encoded. Unexpectedly, the N-terminal peptide for CDR2L, the major antigen in anti-Yo paraneopalstic syndrome, was the most enriched peptide by PhIP-Seq in both technical replicates (**Figure 2A**) (13). Two additional CDR2L peptides were also detected but less enriched, as was the N-terminal peptide of CDR2 (**Figure 2B**). Peptides to other known paraneoplastic autoantigens were not enriched (**Figure 2A** and **Supplementary Table 2**).

SCREENING FOR ANTI-YO ANTIBODIES BY TISSUE-BASED ASSAY

Routine clinical testing for anti-Yo antibodies is often performed by indirect immunofluorescent immunostaining of non-human primate cerebellar tissue. If the immunofluorescence assay (IFA) is positive, reflex immunoblotting of recombinant CDR2 is performed. Because CDR2L and CDR2 peptides were enriched by PhIP-Seq, we screened this patient's CSF for Purkinje cell immunoreactivity by sagittal mouse brain tissue-based assay. As a positive control, we used CSF from a previously reported case of clinically diagnosed anti-Yo paraneoplastic cerebellar degeneration (PCD CSF) that enriched multiple CDR2L peptides by PhIP-Seq [patient 03, (7)] (**Figure 2B**). At a 1:25 dilution, control PCD CSF robustly immunostained cerebellar Purkinje cells as well as sparse cerebral and hippocampal neurons (**Figure 2C** and **Supplementary Figure 2**).

In contrast, CSF from our patient with paraneoplastic myelopathy (PNM CSF) did not immunostain Purkinje cells when performed in technical triplicate and assessed by epifluorescence microscopy at a 1:4 dilution, consistent with the negative clinical testing (**Figure 2C**). Likewise, PNM CSF did not immunostain murine Purkinje cells even after antigen retrieval (**Figure 2C**). However, in one replicate PNM CSF did immunostain hippocampal neurons in a pattern similar to PCD CSF, albeit more sparsely (**Figure 2C**). We further characterized immunopositive replicates by confocal microscopy and found that immunostained hippocampal neurons were morphologically similar to those immunostained by PCD CSF, and that previously undetectable Purkinje cells were also faintly immunostained (**Figure 2C**).

We next performed coimmunostaining with antibodies that have been validated as specific to CDR2L and CDR2. In the cerebellum, the control PCD CSF colocalized with CDR2L and CDR2 staining in Purkinje cells. However, only anti-CDR2L staining co-localized with the control PCD CSF immunostaining of neurons in the molecular layer of the cerebellum. Likewise, PCD CSF and the anti-CDR2L antibody colocalized with hippocampal immunostaining. In contrast, hippocampal CDR2 expression was limited to arteriolar smooth muscle cells, consistent with CDR2 expression as annotated in the Human Protein Atlas (**Supplementary Figure 2**; http://www.proteinatlas.org) (14).

Mouse and human CDR2L are 93% similar by amino acid sequence, however some of the sequence differences are within the peptide that was most enriched by PNM CSF by PhIP-Seq (**Figure 2B**). To ensure the absence of immunostaining was not due to species differences, we co-immunostained human cerebellar tissue with PNM CSF or PCD CSF and anti-CDR2L or anti-CDR2 antibodies. As with mouse, PNM CSF at a 1:4 dilution did not appreciably immunostain human Purkinje cells while PCD CSF was strongly reactive at a 1:25 dilution and colocalized with CDR2L but not CDR2 (**Figure 2D**).

Taken together, these data suggest that some anti-Yo antibodies are not readily detected by Purkinje cell immunostaining—the standard initial screen for anti-Yo antibodies. Moreover, extracerebellar immunostaining may

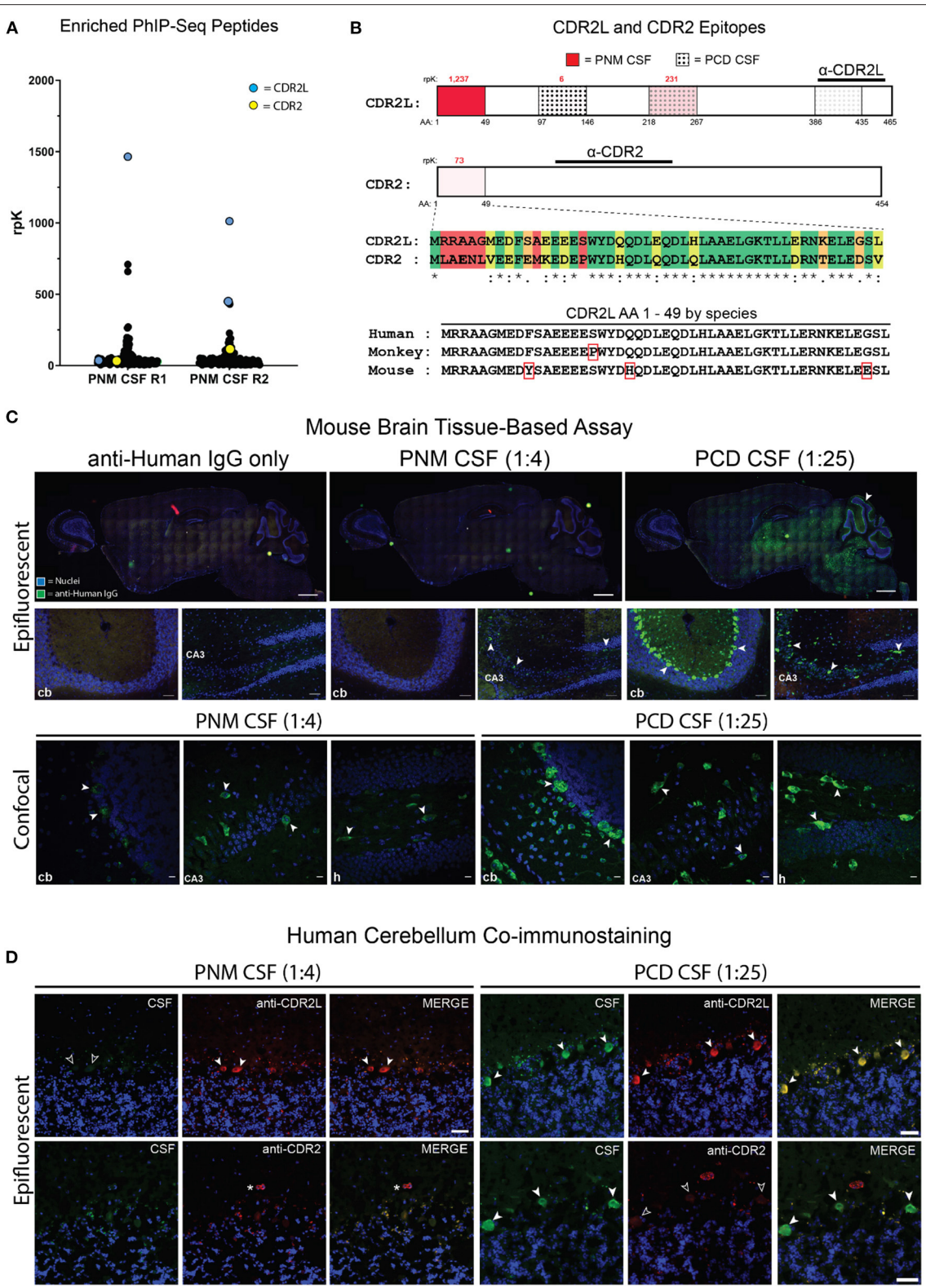

FIGURE 2 | Identification of anti-CDR2L and anti-CDR2 antibodies and PNM CSF tissue-based assay. **(A)** Dot plot of individual PhIP-seq-identified peptides that were enriched in both technical replicates at least 10-fold above reference samples (R1 and R2, replicates 1 and 2, respectively). The most enriched peptide in each replicate mapped to CDR2L (blue points). A CDR2 peptide (yellow) was also identified. PhIP-Seq data for all other enriched peptides (black) are available in **Supplementary Table 2**. **(B)** CDR2L and CDR2 proteins and relevant epitopes. The intensity of red shading is proportional to the enrichment of that peptide by PNM

(Continued)

FIGURE 2 | CSF and the rpK are in red above each of the 3 detected CDR2L peptides and the single enriched CDR2 peptide. The dotted regions (3 rightmost) indicate the peptides that were enriched by PCD CSF whereby density of the dots represents the degree of enrichment. The immunogens for CDR2L and CDR2 commercial antibodies used for immunostaining are indicated by horizontal lines. A sequence alignment for the first 49 amino acids for CDR2L and CDR2 is shown [green, identical amino acids (AA); yellow, chemically similar AA; orange, minimally similar AA; and red, dissimilar AA]. The sequence alignment for the first 49 amino acids of human, monkey, and murine CDR2L are shown. Red rectangles indicate AA that differ from human. **(C)** Epifluorescent and confocal imaging of murine brain tissue immunostained with PNM CSF at 1:4 or PCD CSF at 1:25. cb, cerebellum; CA3, cornu Ammonis 3 of the hippocampus. Scale bars = 1,000 μm for whole brain sagittal images, 50 μm for epifluorescent hippocampal and cerebellar images, and 10 μm for confocal images. **(D)** Epifluorescent images immunostaining of human cerebellum by PNM and PCD CSF at 1:4 and 1:25, respectively. Arrows indicate Purkinje cells (PCs). In the upper left set of images, PNM CSF failed to immunostain CDR2L immunostained PCs (unfilled arrows). On the lower right, anti-CDR2 failed to immunostain PCs immunostained by PCD CSF (unfilled arrows). The asterisk indicates a blood vessel immunostained by anti-CDR2. Scale bars = 50 μm.

indicate the presence of anti-Yo antibodies, even in the absence of Purkinje cell immunoreactivity.

VALIDATION OF ANTI-YO ANTIBODIES IN PATIENT CSF

Because PNM CSF was not immunoreactive to Purkinje cells, we tested for anti-Yo antibodies by western blot. HEK 293T cells were either untransfected or transfected with C-terminal flag-tagged CDR2L (CDR2L-FLAG), CDR2 (CDR2-FLAG), or FIP1L1 (FIP1L-FLAG) as a non-target negative control. HEK 293T cell lysates were separated by SDS-PAGE and probed with PNM CSF at a 1:250 dilution and a commercial anti-FLAG antibody. PNM CSF IgG bound CDR2L and CDR2, but not untransfected HEK293T cell lysate or FIL1LP1-FLAG (end point dilution of 1:1,000 for PNM CSF not shown) (**Figure 3A**).

To further validate anti-CDR2L and anti-CDR2 autoantibodies, we tested PNM CSF for anti-Yo antibodies by HEK 293T overexpression CBA. HEK 293T cells were mock transfected, transfected with CDR2L-FLAG, or transfected with CDR2-FLAG, and immunostained with either PNM CSF (1:10) or PCD CSF as a positive control (1:50). Consistent with our immunoblot results, both PNM and PCD CSF immunostained CDR2L and CDR2-overexpressing HEK 293T CBAs (end point dilution of 1:100 for PNM CSF not shown) (**Figures 3B,C**).

Therefore, despite nearly undetectable immunostaining of Purkinje cells, western blot and CBA demonstrated the presence of anti-CDR2L and anti-CDR2 autoantibodies in PNM CSF.

CDR2L EXPRESSION IS RESTRICTED TO HER2+ CELLS IN PATIENT BREAST CARCINOMA TISSUE

HER2 is overexpressed in up to 96% of breast cancers from anti-Yo PCD patients compared to 15–20% of all breast cancers (15, 16). Additionally, CDR2L is expressed in up to 100% of ovarian carcinomas from patients with anti-Yo PCD and is overexpressed relative to control ovarian carcinoma tissue (17). Consistent with these molecular associations, we found that CDR2L and HER2 were expressed in the same anatomic regions when comparing serial sections of the patient's breast carcinoma tissue (**Figure 3D**). To determine whether CDR2L expression in our patient's tissue was restricted to HER2-expressing cells, we optimized CDR2L and HER2 for dual chromophore immunohistochemistry on control human cerebellum and control HER2+ breast carcinoma tissue from a patient not known to have anti-Yo PCD. We found that CDR2L expression in our patient's breast carcinoma colocalized with, and was restricted to, HER2+ carcinoma cells (**Figure 3E**).

CDR2L AND CDR2 PROTEIN EXPRESSION IN MURINE AND HUMAN SPINAL CORD

Given that our patient presented with a myelopathy, we next asked whether PNM CSF anti-Yo antibodies bind to spinal cord tissue. We first immunostained mouse spinal cord sections with PCD CSF, PNM CSF, and anti-CDR2L and anti-CDR2 antibodies. As with cerebellar tissue, PNM CSF did not immunostain murine spinal cord above background fluorescence. In contrast, PCD CSF immunostained large cells along the longitudinal axis of the murine spinal cord (**Figure 4A**).

According to the Human Protein Atlas, CDR2L gene expression is higher than CDR2 in the human spinal cord; however, to our knowledge, CDR2L and CDR2 proteins have not been characterized in the murine or human spinal cord. In the murine spinal cord, PCD CSF immunostaining was restricted to the cytoplasm of large cells. In contrast, CDR2L immunostaining was both cytoplasmic and nuclear; however, all PCD CSF-immunostained cells were co-stained with CDR2L and vice versa (**Figure 4B**). In contrast, the anti-CDR2 antibody was poorly immunoreactive to neural cells but again strongly stained arteriolar smooth muscle cells (**Figure 4B**). In the human spinal cord, PCD CSF immunostaining also colocalized with anti-CDR2L more so than with anti-CDR2 immunostaining (**Figure 4C**). As with other tissue-based assays, PNM CSF failed to immunostain human spinal cord (data not shown).

FOLLOW-UP AND OUTCOMES

Symptomatically and radiologically, the patient responded to IVIg treatment initially, but she had worsening symptoms 17 months later. Given the newly found thoracic myelopathy, which was suspected to be inflammatory in origin, she was switched to monthly pulsed dose steroids with subsequent stabilization. Small studies evaluating plasma exchange, IVIg, high dose corticosteroids, cyclophosphamide, and rituximab have been conducted in anti-Yo PCD, and no single agent has shown consistent efficacy (18). Furthermore, a case series of isolated paraneoplastic myelopathy associated with autoantibodies other than anti-Yo responded poorly to immunotherapy resulting

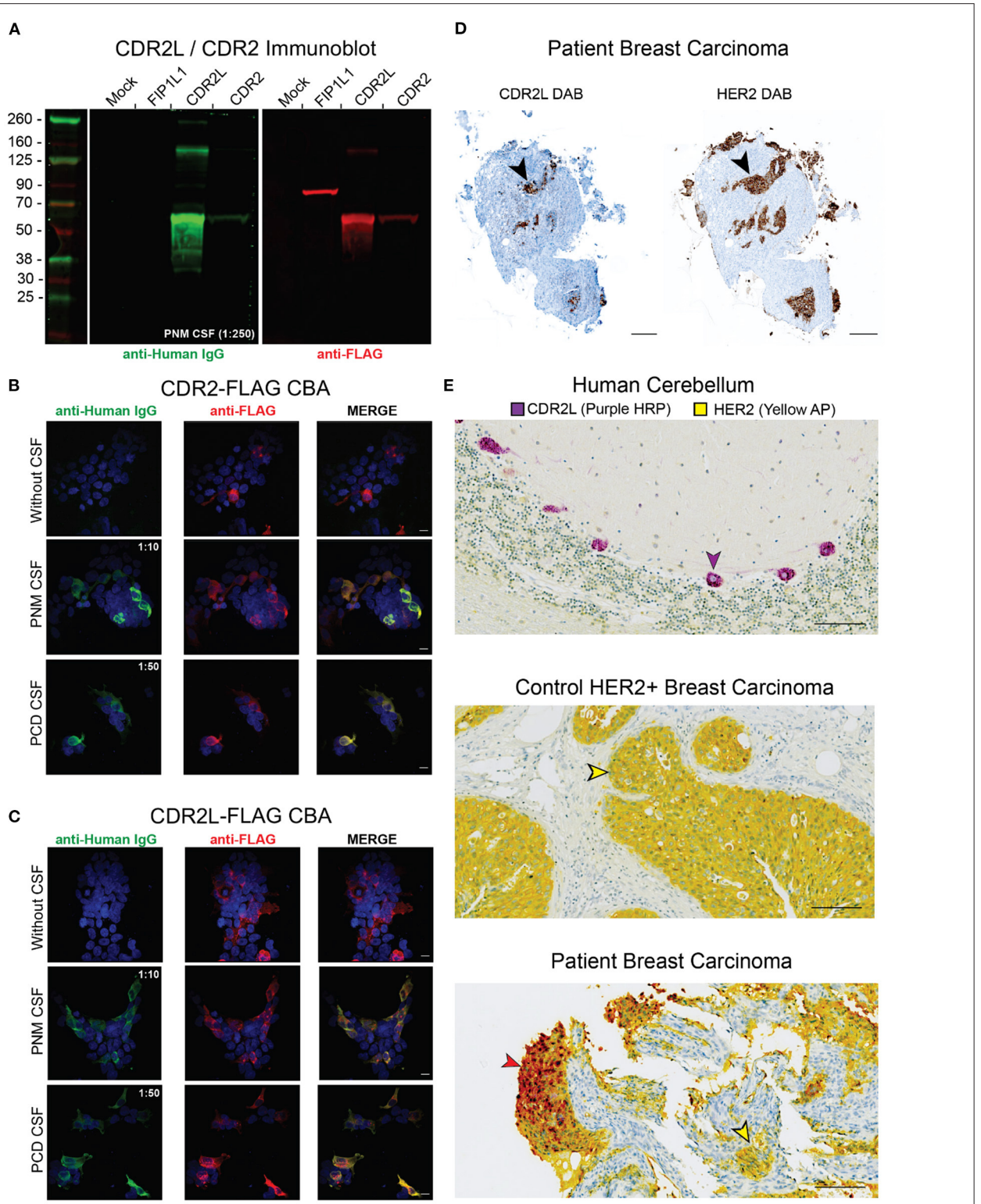

FIGURE 3 | Validation of anti-Yo antibodies in PNM CSF. **(A)** HEK293T cells were transfected with FIP1L1-FLAG, CDR2L-FLAG, or CDR2-FLAG. Lysates were separated by SDS-PAGE and resulting immunoblots were probed with PNM CSF (green, 1:250) and counterstained with an anti-Human IgG 800CW secondary antibody (green). The membrane was the probed with anti-FLAG and anti-rabbit 680RD secondary antibody (red). PNM CSF IgG recognized recombinant CDR2L and CDR2 but not FIP1L1. **(B)** HEK293T cells were transfected with CDR2-FLAG and immunostained with PNM CSF at 1:10, PCD CSF at 1:50 (both green) and an

(Continued)

FIGURE 3 | anti-FLAG antibody (red). Scale bars are 10 μm. **(C)** HEK293T cells were transfected with CDR2L-FLAG and immunostained with PNM CSF at 1:10, PCD CSF at 1:50 (both green) and an anti-FLAG antibody (red). Scale bars are 10 μm. **(D)** 3,3′-Diaminobenzidine (DAB) immunohistochemistry of serial sections of the patient's breast carcinoma tissue for CDR2L (left) and HER2 (right). Black arrowheads highlight a corresponding area of DAB staining on serial sections. Scale bars = 200 μm. **(E)** Dual chromophore immunohistochemistry for CDR2L (purple HRP, horse radish peroxidase) and HER (yellow AP, alkaline phosphatase) on control cerebellum (top), control HER2 breast tissue (middle), and the patient's breast carcinoma (bottom). The purple arrowhead indicates a CDR2L+/HER2– Purkinje cell in the top panel. The yellow arrowheads indicate CDR2L–/HER2+ cells in control breast carcinoma (middle) and the patient's breast carcinoma (bottom). The brick red arrow indicates CDR2L+/HER2+ cells in the patient's breast carcinoma (bottom). Red results from the co-localization of the yellow and purple chromogens. Scale bars = 100 μm.

in significant disability (19). Therefore, her mild improvement with immunotherapy was atypical for isolated paraneoplastic myelopathy. Her improvement also coincided with the remission of her breast cancer. Given that our patient had completed anti-tumor therapy and demonstrated a potential response to IVIg and corticosteroids, we transitioned her to rituximab as a steroid-sparing agent.

DISCUSSION

Here we presented a woman with a history of breast cancer and antibodies against anti-Yo antigens CDR2L and CDR2 despite negative clinical testing. Nearly 40% of PCD cases test negative for previously classified autoantibodies, and seronegative paraneoplastic myeloneuropathy has been reported in breast cancer (20). Serum and CSF that screen positive for anti-Yo antibodies by tissue-based immunostaining but only have anti-CDR2L antibodies (not anti-CDR2) are at risk for a false negative result because only CDR2 is included in confirmatory reflex assays (13, 21, 22). In this instance, initial screening of our patient's CSF for PND autoantibodies by IFA was negative, so presumably no confirmatory reflex assays looking for antibodies to either CDR2 or CDR2L were performed. Moreover, in contrast to many clinically diagnosed anti-Yo syndromes, our patient's CSF lacked oligoclonal bands and had a normal cell count, protein level, and IgG index (23). By maintaining a high suspicion for an inflammatory condition and applying unbiased high-resolution whole human proteome PhIP-Seq, we identified CDR2L and CDR2 antibodies in the CSF that we subsequently confirmed by immunoblot and CBA.

These data suggest that anti-Yo antibody detection depends on whether and how the target epitopes are represented in the diagnostic assays. In our patient's case, PhIP-Seq, immunoblot, and CBA were more sensitive than rodent brain immunohistochemistry. These findings may suggest that some CDR2 and CDR2L epitopes are inaccessible *in situ* because they are buried within a folded protein or shielded by protein-protein interactions. Indeed, other research studies have identified anti-Yo PND cases whose CSF screened negative for CDR2L/CDR2 antibodies by IFA (24, 25). Although we were able to detect anti-Yo antibodies in our patient's CSF at a 1:100 and 1:1,000 dilution by overexpression CBA and immunoblot, respectively, we cannot rule out that the antibody titer was below the limit of detection for tissue-based IFA (<1:240 for serum or <1:2 for CSF based on clinical testing parameters) where the effective concentration of CDR2L and CDR2 may be lower. In either case, our patient highlights the potential for false negatives when using tissue staining as a threshold assay.

Most cases of PNM are associated with antibodies against amphiphysin, Hu, or CV2/CRMP5, while anti-Yo antibodies are typically associated with PCD (19). Anti-Yo PCD with superimposed myelopathy is uncommon (24, 26–28) and isolated anti-Yo myelopathy [(24), case 7] or myeloneuropathy rarer still (10). It is unknown why some anti-Yo patients lack obvious cerebellar findings on exam though this does not preclude subclinical cerebellar dysfunction. This is certainly possible in the patient reported here though the structural imaging of the cerebellum was normal, and it was felt that the extensive dorsal column lesion in the thoracic cord accounted for her neurologic deficits.

In anti-Yo PCD, malignant expression of CDR2L and CDR2 elicits an immunologic response as evidenced by B and T cell inflammatory tumor infiltrates and CDR2L protein deposits in tumor-associated tertiary lymphoid structures (17). Endogenous Purkinje cell expression of CDR2L and CDR2 provides a direct pathogenic link, as inflammatory lymphocytes have been documented in post-mortem cerebellar tissue from patients with anti-Yo PCD (29). Demyelination and vacuolization of spinal cord tissue has been reported in a patient with anti-Yo PCD (29), but to our knowledge CDR2L and CDR2 protein expression in the human spinal cord and the myelopathology of anti-Yo PNM have not been further characterized. We detected CDR2L protein expression in the murine and human spinal cord that colocalized with anti-Yo antibodies from a patient with PCD. In contrast, CDR2 expression was primarily limited to arterioles and was poorly colocalized with anti-Yo antibodies. This finding is concordant with the growing literature suggesting that CDR2L is likely the pathogenic antigen in anti-Yo PNDs generally (7, 13, 24). Thus, the pathogenic mechanism of anti-Yo PNM is likely similar to anti-Yo PCD. Indeed, consistent with tumors from patients with anti-Yo PCD, CDR2L was highly expressed in our patient's HER2+ breast carcinoma tissue. The degree of dysfunction in different tissues in individual patients may reflect variation in MHC peptide presentation in different anatomic regions (30).

Standard clinical autoantibody testing fails to identify a diagnostic antibody in up to one third of patients with PNDs, some of whom may harbor undetected classified autoantibodies (25). Few laboratories use multiple modalities upon initial screening for paraneoplastic autoantibodies, and single modality screening is known to risk false positive and negatives for some antibodies including anti-Yo (31). We previously showed that PhIP-Seq significantly enriched CDR2L and/or CDR2 peptides in

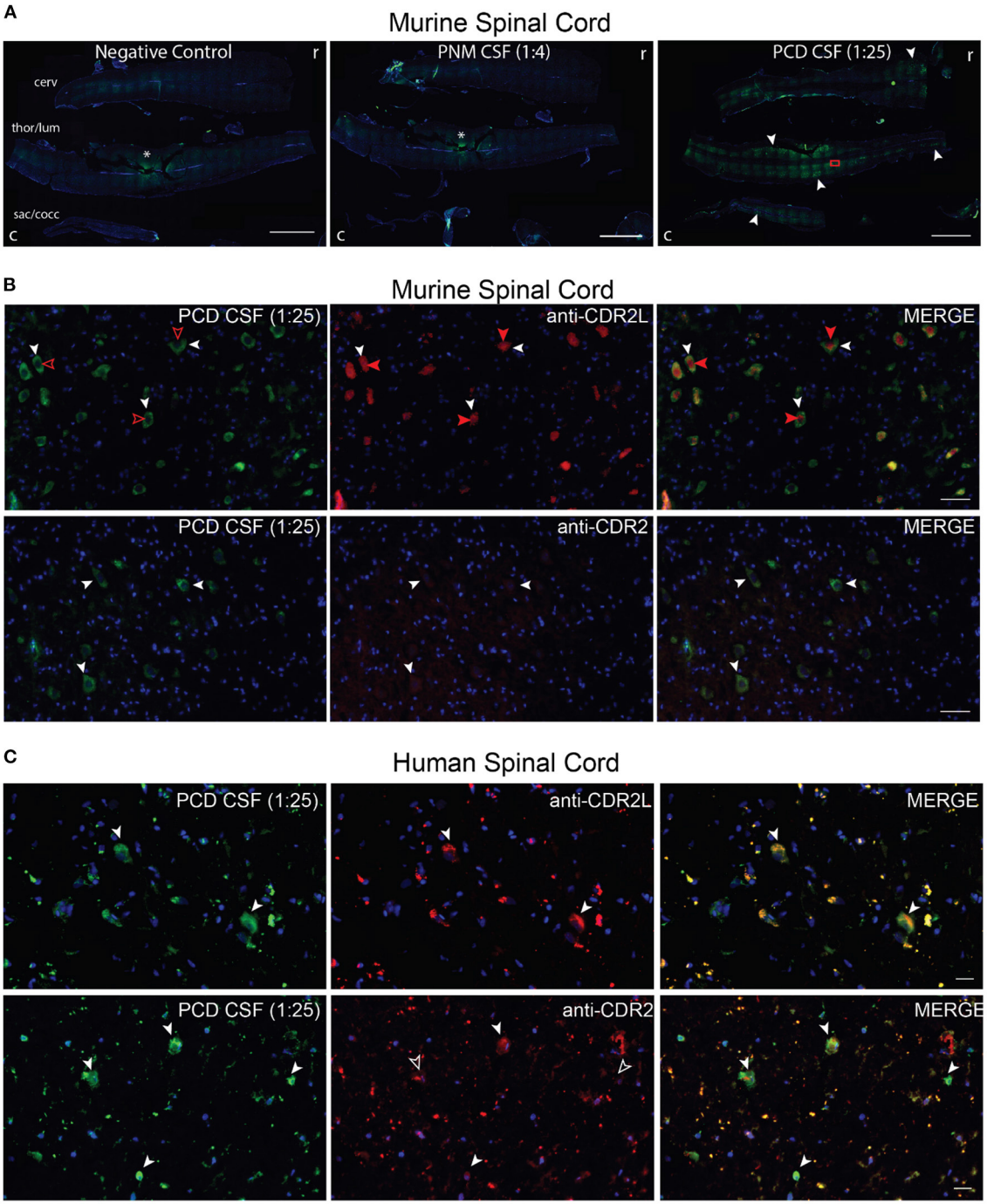

FIGURE 4 | PNM CSF, PCDF CSF, CDR2L and CDR2 immunostaining of spinal cord. **(A)** Cervical (cerv), thoracolumbar (thor/lum), sacrococcygeal (sac/cocc) murine spinal cord was immunostained with PNM CSF (1:4) or PCD CSF (1:25). The rostral (r) and caudal (c) ends are on the right and left of each image, respectively. PNM CSF did not immunostain murine spinal tissue above background (asterisk = tissue artifact also seen in the negative control). PCD CSF immunostained cells along the longitudinal axis of the spinal cord (filled arrows). The red rectangle indicates the approximate region of images shown in **(B)**. Scale bars = 2,000 μm. **(B)** Coimmunostaining of murine spinal cord with PCD CSF (1:25) and anti-CDR2L or anti-CDR2. PCD CSF and anti-CDR2L immunostained the cytoplasm of large cells and colocalized with cytoplasmic anti=CDR2L immunostaining (white arrowheads). However, anti-CDR2L also immunostained nuclei (filled red arrows) but PCD CSF did not (unfilled red arrowheads). Scale bars = 200 μm. **(C)** Coimmunostaining of human spinal cord with PCD CSF. PNM CSF failed to immunostain human spinal cord (not shown). PCD CSF immunostained sparse cells that were also CDR2L positive (top row, white arrowheads). In the lower row, PCD CSF cells are costained by anti-CDR2L (white arrowheads). In the lower row, only some cells immunostained by PCD CSF were also immunostained by anti-CDR2 (filled arrow heads, co-stained cells; unfilled, PCD CSF exclusive cells). Scale bars = 20 μm.

all 36 screened cases of clinically diagnosed anti-Yo PCD (51 of 53 biospecimens) with no difference in sensitivity between serum and CSF (7). This study extends those data to show that PhIP-Seq may identify paraneoplastic cases for which tissue-based assays are insufficiently sensitive. Together with prior literature, our data suggests a need for complementary autoantibody testing to account for differential target epitope availability among diagnostic tests.

PATIENT PERSPECTIVE

Our patient noted that having undiagnosed progressive symptoms increased her general sense of anxiety. She was relieved when immunosuppression provided some relief and was grateful for the opportunity to participate in research that helped clarify the likely cause of her disease.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UCSF Human Research Protection Program. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by UCSF Office of Research Institutional Animal Care and Use Program. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

CB, TN, and BA performed the laboratory experiments. NP and MK evaluated the patient, and KZ and JA enrolled the patient. MP interpreted the tumor pathology. SP and MW supervised the study and data analysis. CB, NP, SP, and MW wrote the first draft of the paper. All authors provided critical editing of the text.

FUNDING

Confocal microscopy with the CSU-W1 spinning disk was supported by the S10 Shared Instrumentation grant (1S10OD017993-01A1). Tumor histology was supported in part by the University of California, San Francisco Histology & Biomarker Core, a subgroup of the Helen Diller Family Comprehensive Cancer Center Biorepository and Tissue Biomarker Technology Core (BTBMT). The BTBMT was supported by the National Cancer Institute of the National Institutes of Health under Award Number P30CA082103. This work was further supported by: NIMH R01MH122471 (SP, MW, and KZ), NINDS 2R25NS070680-11 (NP), NINDS K08NS096117 (MW), Brain Research Foundation (SP). CB was supported by a Hanna H. Gray Fellowship, Howard Hughes Medical Institute, President's Postdoctoral Fellowship Program, the University of California, the John A. Watson Scholar Program, the University of California, San Francisco, and the Latinx Center of Excellence Grant no. D34HP3178.

ACKNOWLEDGMENTS

We thank Dr. Tibor Krenács for his guidance on immunostaining acetone fixed human tissue. We thank Delaine Larsen, Kari Herrington, and SoYeon Kim of the University of California, San Francisco Nikon Imaging Center for their imaging support. We thank Jennifer Bolen, Scott VandenBerg, and Kristine Wong for their help in preparing and interpreting the tumor histology. We thank the patient for her participation in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.728700/full#supplementary-material

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Conflict of Interest: MW receives unrelated research support from Roche/Genentech.

The remaining 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.

frontiers in Immunology

ORIGINAL RESEARCH
published: 24 November 2021
doi: 10.3389/fimmu.2021.738097

Risk Factors and Brain Metabolic Mechanism of Sleep Disorders in Autoimmune Encephalitis

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Background: Sleep disorders (SDs) in autoimmune encephalitis (AE) have received little attention and are poorly understood. We investigated the clinical characteristics, risk factors, and cerebral metabolic mechanism of SD in AE.

Methods: Clinical, laboratory, and imaging data were retrospectively reviewed in 121 consecutively patients with definite AE. The risk factors for SD in AE were estimated by logistic regression analysis. Group comparisons based on ^{18}F-fluorodeoxy-glucose positron emission tomography (^{18}F-FDG-PET) data were made between patients with and without SD, to further analyze potential brain metabolic mechanism of SD in AE.

Results: A total of 52.9% patients (64/121) with SD were identified. The multivariate logistic model analysis showed that smoking [odds ratio (OR), 6.774 (95% CI, 1.238–37.082); $p = 0.027$], increased Hamilton Depression scale (HAMD) score [OR, 1.074 (95% CI, 1.002–1.152); $p = 0.045$], hyperhomocysteinemia [OR, 2.815 (95% CI, 1.057–7.496); $p = 0.038$], elevated neuron-specific enolase (NSE) level [OR, 1.069 (95% CI, 1.007–1.135); $p = 0.03$] were independently correlated with higher risk of SD in AE patients. Contrastingly, high MoCA score [OR, 0.821 (95% CI, 0.752–0.896); $p < 0.001$] was associated with lower risk of SD in AE subjects. Compared to controls, AE patients had less total sleep time, less sleep efficiency, longer sleep latency, more wake, higher percent of stage N1, lower percent of stage N3 and rapid eye movement, and more arousal index in non-rapid eye movement sleep ($p < 0.05$ for all). Voxel-based group comparison analysis showed that, compared to patients without SD, patients with SD had increased metabolism in the basal ganglia, cerebellum, brainstem, median temporal lobe, thalamus, and hypothalamus [$p < 0.05$, false discovery rate (FDR) corrected]; decreased metabolism in superior frontal gyrus, medial frontal gyrus, and posterior cingulate cortex ($p < 0.001$, uncorrected). These results were confirmed by region of interest-based analysis between PET and sleep quality.

Conclusion: Smoking, increased HAMD score, hyperhomocysteinemia, and elevated NSE level were correlated with higher risk of SD. High MoCA score was associated with

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Edited by:
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University of Ulsan, South Korea

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

Received: *08 July 2021*
Accepted: *08 November 2021*
Published: *24 November 2021*

Citation:
Liu X, Yu T, Zhao X, Yu P, Lv R, Wang C, Ai L and Wang Q (2021) Risk Factors and Brain Metabolic Mechanism of Sleep Disorders in Autoimmune Encephalitis. Front. Immunol. 12:738097. doi: 10.3389/fimmu.2021.738097

lower risk of SD in AE subjects. Moreover, a widespread metabolic network dysfunction may be involved in the pathological mechanism of SD in AE.

Keywords: autoimmune encephalitis, sleep disorders, risk factors, positron emission tomography, brain metabolism, pathological mechanism

INTRODUCTION

In most patients of autoimmune encephalitis (AE), the common clinical features include seizures, cognitive deficits, psychosis, and abnormal behaviors (1). In addition to these classical characteristics, there are other significant symptoms that have not been described in detail, such as sleep disorders (SDs). Although some studies recently demonstrated that SD were relatively frequent, and could lead to a poor prognosis for AE patients, SDs in patients with AE have still not received more attention (2–4). Recent studies have shown that the sleep features of AE mainly include insomnia, hypersomnolence, rapid eye movement (REM) sleep behavior disorder (RBD), and sleep apnea (5–9). However, only a limited number of cases indicate SD characteristics of AE. Moreover, the risk factors and potential pathological mechanisms of SD in AE remain unknown. Prior studies have investigated that SD tend to occur in neurological diseases, such as stroke, neurogenerative disorders (10, 11). Nevertheless, little information about risk factors of SD is available for AE patients; therefore, we hypothesized that common risk factors would be associated with SD of AE.

Furthermore, regarding pathological mechanism of SD, neuroimaging methods can be used to clarify whether SDs are related to corresponding alterations in brain structure or functional activity. ^{18}F-fluoro-2-deoxy-d-glucose positron emission tomography (^{18}F-FDG-PET) is a functional imaging modality for *in vivo* evaluation of the pathophysiology of the brain *via* application of ^{18}F-FDG (12). Previous studies have shown that ^{18}F-FDG-PET has a high sensitivity in the diagnosis of AE patients (13, 14). Meanwhile, patients with sleep disturbances showed abnormal metabolism in the brain regions that modulate sleep (15, 16). However, to the best of our knowledge, there is no systematically relevant study to evaluate the brain metabolic mechanisms of SD in patients with AE. With the aim of recognizing SD in AE, we reported 121 patients with a definite diagnosis of autoimmune encephalitis, focusing on the risk factors and brain metabolic mechanism of SD in AE.

Abbreviations: AE, autoimmune encephalitis; SD, sleep disorders; 18F-FDG-PET, 18F-fluorodeoxy-glucose positron emission tomography; NMDAR, N-methyl-D-aspartate receptor; LGI1, leucin-rich glioma inactivated-1; CASPR2, contactin-associated protein-2; GABAB, γ-aminobutyric acid type B; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; GAD65, glutamic acid decarboxylase 65; PSQI, Pittsburgh sleep quality index; BMI, body mass index; MoCA, Montreal cognitive assessment; HAMD, Hamilton Depression scale; HAMA, Hamilton Anxiety scale; NLR, neutrophil-to-lymphocyte ratio; NSE, neuron-specific enolase; SPM, statistical parametric mapping; FDR, false discovery rate; IQR, interquartile range.

MATERIALS AND METHODS

Patients

This study was approved by the Ethics Committee of Beijing Tiantan Hospital that was affiliated to the Capital Medical University of the People's Republic of China. The study was conducted in accordance with the Declaration of Helsinki, and all patients provided informed consent for the use of their medical records.

Patients were consecutively recruited and retrospectively analyzed from October 2014 to June 2021 at the Department of Neurology, Beijing Tiantan Hospital, Capital Medical University. The inclusion criteria in this study were as follows. First, patients had definite autoimmune encephalitis; the clinical diagnosis of definite AE should meet the following criteria: (a) subacute onset (rapid progression of <3 months) of working memory deficits, seizures, or psychiatric symptoms suggesting involvement of the limbic system; (b) bilateral brain abnormalities on MRI highly restricted to the medial temporal lobes; (c) cerebrospinal fluid (CSF) pleocytosis (white blood cell count of more than five cells per mm^3); and (d) electroencephalogram (EEG) with epileptic or slow-wave activity involving the temporal lobes. The diagnosis can be made when all four criteria have been met, and final diagnosis was confirmed by the detection of serum or CSF positive for specific neuronal autoantibodies, including classical paraneoplastic antibodies (Hu, Yo, Ri, Ma2, CV2, and Amphiphysin), N-methyl-D-aspartate receptor (NMDAR), leucin-rich glioma-inactivated-1 (LGI1), contactin-associated protein-2 (CASPR2), γ-aminobutyric acid type B receptor ($GABA_BR$), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), and glutamic acid decarboxylase 65 (GAD65). Serum and CSF samples were tested using both cell-based assays (Euroimmun, Lübeck, Germany) and immunohistochemical analyses in the Neuroimmunology Laboratory of the Peking Union Medical College Hospital (Beijing, China). Second, patients should be first admitted in our department from symptom onset. The exclusion criteria included (1) patients did not fulfill sleep assessment or received medication treatments before sleep assessment; (2) patients missed ^{18}F-FDG-PET data; (3) patients had concurrent autoantibodies (two or more); and (4) patients had history of sleep disorder before disease onset.

Data Collection

Clinical information on demographics, medical history, smoking, drinking, days from onset to diagnosis, comorbid symptoms, neuropsychological and psychiatric test, accompanied tumor, in-hospital laboratory test, electroencephalogram (EEG) and imaging examination, and treatments, were obtained from electronic

medical records at first admission. Body mass index (BMI) was calculated as weight (kg)/height (m^2). Neuropsychological test contained Montreal Cognitive Assessment (MoCA) (17). Depression and anxiety were evaluated using the Hamilton Depression scale (HAMD) and the Hamilton Anxiety scale (HAMA). Laboratory test included specific antibodies; white blood cell counts, proteins, and oligoclonal bands in CSF; homocysteine (hyperhomocysteinemia was defined as homocysteine concentration > 15 μmol/L); neutrophil-to-lymphocyte ratio (NLR) was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count; neuron-specific enolase (NSE); and serum sodium. Imaging examination included magnetic resonance imaging (MRI) and ^{18}F-FDG-PET.

Sleep Assessment

Sleep assessment was performed through face-to-face or telephone interview from all patients or their caregivers. First, sleep complaints should be obtained, including insomnia, parasomnia, sleep breathing disorders, sleep-related behaviors or movements, and hypersomnolence. In addition, sleep quality was assessed with the standardized Pittsburgh Sleep Quality Index (PSQI) questionnaire, which evaluates multiple dimensions of sleep over a 1-month period (18). It is a self-reported questionnaire that has seven components, including subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medications, and daytime dysfunction. Each of the seven components is scored according to levels 0–3, and each score is eventually accumulated to yield a total PSQI score ranging from 0 to 21, where the higher the score, the worse the sleep quality. In this study, PSQI scores >5 indicated that patients had SD. Then, included AE patients were further divided into two subgroups (AE with SD and AE without SD) according to their PSQI results.

Only 28 patients underwent nocturnal video-polysomnography (PSG) examinations. PSG was conducted using an integrated digital recording system (Greal, Compumedics, Melbourne, Australia) that contained data acquisition, storage, and sleep analysis at the Sleep Center of Beijing Tiantan Hospital, Capital Medical University, China. Every PSG recording contained multiple channels, mainly including electrooculogram (EOG), electromyogram (EMG), electrocardiogram (ECG), respiratory signals, and EEG. EEG channels covered three brain regions (frontal, central, and occipital). Equipment placement, sleep staging, and event scoring were completed by registered polysomnographic technician. Subsequently, the following PSG parameters were collected: total time in bed; total sleep time (TST); sleep latency; sleep efficiency; wakefulness after sleep onset; percentage of stage N1, N2, N3, and rapid eye movement (REM); arousal index (AI) in whole sleep, non-rapid eye movement (NREM) sleep, and REM sleep; apnea hypopnea index (AHI); limb movements index (LMI); and periodic limb movements index (PLMI). In addition, 18 age- and gender-matched controls were recorded using the same procedures. The above variables were measured by two experienced sleep specialists blinded to clinical information of the conditions of either patients or controls, and in case of obvious discordance in their initial evaluations, an informed consensus statement was reached. The Kappa coefficient of two specialists was 0.82. REM sleep behavior disorder (RBD) was defined as a parasomnia with dream-enactment behaviors occurring during REM sleep and associated with the lack of the physiological REM sleep muscle atonia. Obstructive sleep apnea (OSA) was defined as an AHI of five or more events per hour.

^{18}F-FDG-PET Procedure and Quantitative Analysis

All raw data of PET images were collected from PET/CT workstation; a detailed ^{18}F-FDG-PET procedure has been published elsewhere using a PET/CT scanner (Elite Discovery, GE HealthCare, USA) (19). Before examination, patients did not receive neuroleptic drugs, fasted for at least 6 h, and controlled fasting blood glucose levels <8 mmol/L. Then, patients were injected with ^{18}F-FDG at a dose of 3.7–5.0 MBq/kg in a quietly dedicated room. After a 45–60-min uptake period, brain PET scan was performed in the 3D-time-of-flight mode for 10 min, and whole-body PET scan was performed for approximately 20–30 min. The brain imaging data were reconstructed using ordered subset expectation maximization methods, with four iterations and eight subsets, and smoothing with a 5-mm full-width at half-maximum filter. Statistical parametric mapping (SPM) 12 (Wellcome Centre for Human Neuroimaging, London, UK) implanted in a MATLAB 2018a environment (MathWorks, Inc., USA) was used for imaging reprocessing analysis. The preprocessing steps were as follows: first, the PET images were spatially normalized into a common Montreal Neurological Institute (MNI) atlas anatomical space following a 12-parameter affine transformation and nonlinear transformations, yielding images composed of 2 mm × 2 mm × 2 mm voxels. Then, default SPM smoothing was applied using a 14-mm Gaussian kernel to increase the signal-to-noise ratio.

For the analysis on brain metabolic mechanism of sleep disorders in AE, we carried out group-level comparison between AE with SD and without SD by two-sample t-test model of SPM12 with age and gender as the nuisance variables. Significant results were viewed at the height threshold ($p < 0.001$) and corrected for multiple comparisons (FDR, corrected, $p < 0.05$). If no cluster of significant difference was found, the more liberal threshold at $p < 0.001$ uncorrected was considered to perform further exploratory analyses (20). Finally, we used the xjView SPM extension (Cui & Li, Human Neuroimaging Lab, Baylor College of Medicine) to visualize the corresponding anatomic locations of each peak MNI of these significant different clusters.

To further explore homogeneity of regional brain glucose metabolic changes between groups in particular spatial pattern, we performed a volumetric region of interest (ROI) analysis in the significant clusters based on aforementioned SPM results. First, the following 10 ROIs were identified: medial temporal lobe (MTL, mainly including amygdala and hippocampus), posterior cingulate cortex (PCC), basal ganglia (BG, including globus pallidus, putamen, and caudate), brainstem (including midbrain, pons, and medulla), superior frontal gyrus (SFG)

and medial frontal gyrus (MFG), thalamus, hypothalamus, cerebellum anterior lobe (CAL), and cerebellum posterior lobe (CPL). These ROIs were defined based on the group-level results obtained from SPM analysis. The brainstem, hypothalamus, and CAL and CPL regions of interest were generated using the WFU-Pickatlas toolbox for SPM12 based on Talairach Daemon lobars, and the remaining ROIs were derived and summarized from the Anatomical Automatic Labeling (AAL) atlas. Subsequently, the ratio of the standardized uptake value (SUVR) representing ^{18}F-FDG uptake calculates in those selected ROIs was obtained from each individual. Briefly, an SUVR was derived for ^{18}F-FDG-PET from the voxel size weighted median uptake in the regions of interest normalized to the whole brain.

Statistical Analysis

Continuous variables with a normal distribution were presented as the mean ± standard deviation, and non-normal variables were reported as the median [interquartile range (IQR)]. The normality of the data was performed by the Shapiro–Wilk test. Categorical variables were showed as frequency with corresponding percentage. We compared groups using t-tests for continuous variables that were normally distributed, Mann–Whitney U tests for non-parametric data, and χ^2 tests or Fisher's exact tests for categorical variables.

Clinical variables were comprehensively collected for possible inclusion into the risk model. We used binary logistic regression to assess independent risk factors associated with AE with SD; all variables with $p < 0.2$ in the univariate analysis were included in multivariable logistic regression model. Subsequently, the likelihood ratio test was used in a backwards elimination process ($p < 0.05$ to retain, $p > 0.1$ to remove) to select the final set of independent risk factors for retention into the model. Odds ratio (OR) with 95% CI was presented for logistic regression model. The correlation between the SUVR values of selected ROIs and PSG parameters was conducted using Spearman test. A two-sided $p < 0.05$ was considered to indicate statistical significance. SPSS 22.0 software package (IBM Corp., Armonk, New York, USA) and Prism 8 (GraphPad Software, CA, USA) were used for statistical analyses.

RESULTS

Patient Characteristics

The flowchart of patient inclusion is shown in **Figure 1**. A total of 187 patients with definite and antibody-confirmed AE were eligible to participate in this study, of whom 121 patients were ultimately identified in the current study after exclusion, including 19 patients with NMDAR antibodies, 71 patients with LGI1 antibodies, 4 patients with CASPR2 antibodies, 21 patients with $GABA_BR$ antibodies, and 6 patients with GAD65 antibodies. Among these cases, 52.9% of AE patients (n = 64) had SD (36.8% NMDAR, 59.2% LGI1, 75% CASPR2, 47.6% $GABA_BR$, and 33.3% GAD65; **Figure 2A**); the median PSQI score in the group of SD was 9 (IQR, 7–14), which was obviously higher than those without SD [median, 9 (IQR, 7–14) vs. 3 (IQR, 2–3), $p < 0.001$). In addition, patients with CASPR2 and LGI1 had higher PSQI score than those with NMDAR antibodies ($p < 0.05$, **Figure 2B**).

There is no significant difference regarding the median time from diagnosis to the sleep assessment [9 (IQR, 6–20) days vs. 9 (IQR, 6–17) days, $p = 0.892$) between patients with SD and without SD. The baseline characteristics of patients based on sleep quality are summarized in **Table 1**. Patients with SD were older than those without SD (median age, 55.4 vs. 49.0 years, $p = 0.001$). Hypertension, smoking, cognitive deficits, psychosis, and abnormal behaviors showed statistically differences between patients with SD and without SD ($p < 0.05$ for all). Compared with patients without SD, patients with SD had higher levels of MoCA score ($p = 0.001$) and NSE ($p < 0.001$) and had lower levels of serum sodium ($p = 0.001$). Eleven of these patients (9%) were associated with tumors: 8 lung cancers (2 with LGI1 antibodies, 7 with GABAB antibodies), 1 colorectal adenoma (1 with LGI1 antibodies), 1 ovarian teratoma (1 with NMDAR antibodies), and 1 thymoma (1 with LGI1 antibodies). Tumor removal was performed in three patients, and chemotherapy was performed in eight patients. In a univariate analysis, seven patients (10.9%) presented tumors in the group of sleep disorders, and four patients (7%) with tumor were shown in the group of patients without sleep disorders. There was no statistical significance between the two groups ($p = 0.454$). All patients received first-line immunotherapy [20 IV methylprednisolone (IVMP) alone, 21 IV immunoglobulins (IVIg) alone, and 80 IVMP + IVIg]. Twelve patients additionally received second-line immunotherapy (four rituximab and eight mycophenolate mofetil). There was no significant difference between patients with SD and without SD regarding relevant treatments ($p = 0.363$). More details of corresponding descriptions are reported in **Table 1**.

PSG Findings

A subgroup of 28 patients completed PSG recordings. These 28 patients were a representative sample of the whole cohort of 121 patients because there were no significant differences across clinical variables between patients with PSG and the whole cohort (**Supplementary Table S1**).

The sleep parameters of PSG are presented in **Table 2**. The median TST was 292.8 (160–489.4) min. Sleep efficiency was approximately 56.1%. Sleep onset latency was 37 (1.5–189) min. Wakefulness after sleep onset was 145.8 (26.5–264.5) min. For percent of sleep stages, the stage N1, N2, N3, and REM comprised 25.2% (4.3%–56.8%), 51.4% (15.6%–85.6%), 9.4% (0%–29.8%), and 12.5% (0.7%–32.5%) of the TST, respectively. REM sleep latency was 139 (24–287.5) min. Nine patients had dream enhancement behaviors, and the final diagnosis of RBD was confirmed on PSG in five patients (5/28, 17.9%), of whom three had LGI1 antibodies. Sixteen of 28 patients (57.1%) developed OSA with median AHI 10/hour. AI in whole sleep, NREM, and REM were 8.6 (0–33.5), 17 (0.5–33), and 14 (0.9–30), respectively. The median LMI and PLMI were 16/h (1–360/h) and 12/h (0–25/h), respectively.

There were no significant differences between patients with SD and without SD regarding age, gender, and body mass index (**Table 2**). Compared to controls, AE patients had less TST, less

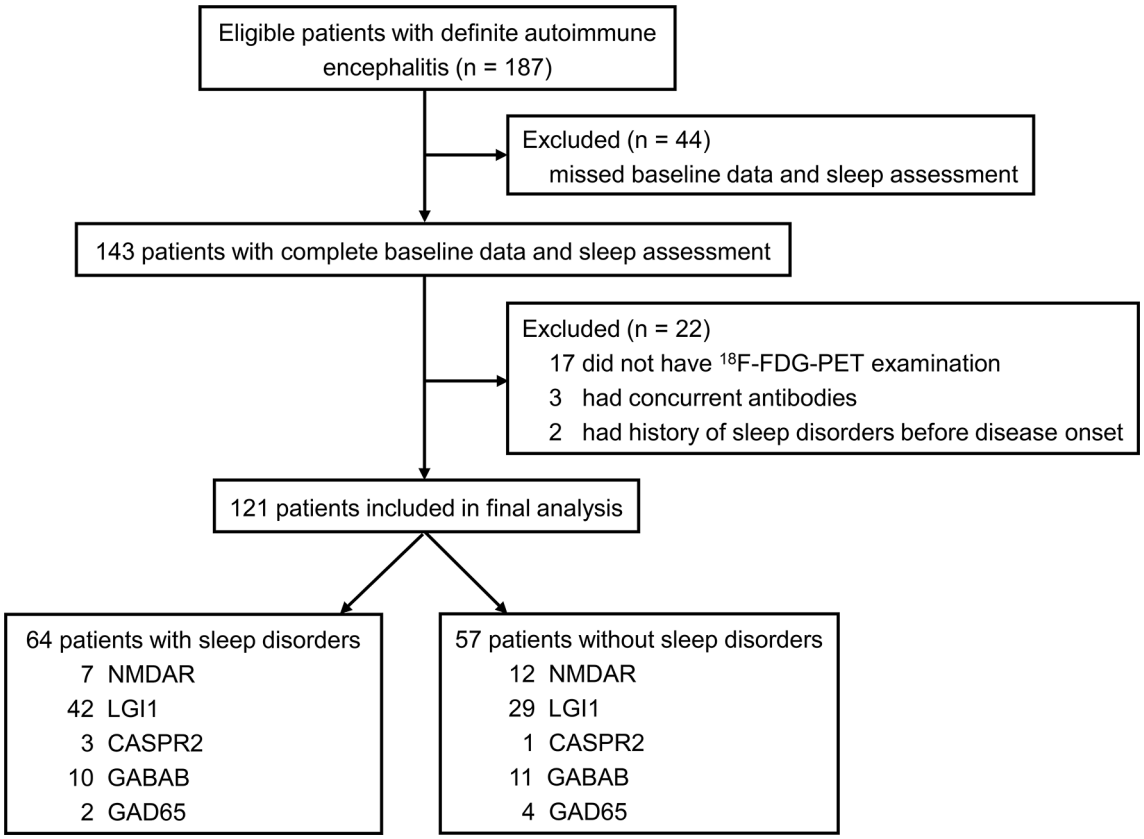

FIGURE 1 | Flowchart of patient selection. ^{18}F-FDG-PET, ^{18}F-fluorodeoxy-glucose positron emission tomography; NMDAR, N-methyl-D-aspartate receptor; LGI1, leucin-rich glioma inactivated-1; CASPR2, contactin-associated protein-2; GABAB, γ-aminobutyric acid type B; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; GAD65, glutamic acid decarboxylase 65.

sleep efficiency, longer sleep latency, more wake, higher percent of stage N1, lower percent of stage N3 and REM, and more AI in NREM sleep ($p < 0.05$ for all). Other sleep variables demonstrated no significant differences between the two groups.

Analysis of Risk Factors for SD in AE

Results of multivariate logistic analysis of the final model are shown in **Table 3**. We selected variables with $p < 0.2$ in the univariate analysis, including age, male, hypertension, smoking,

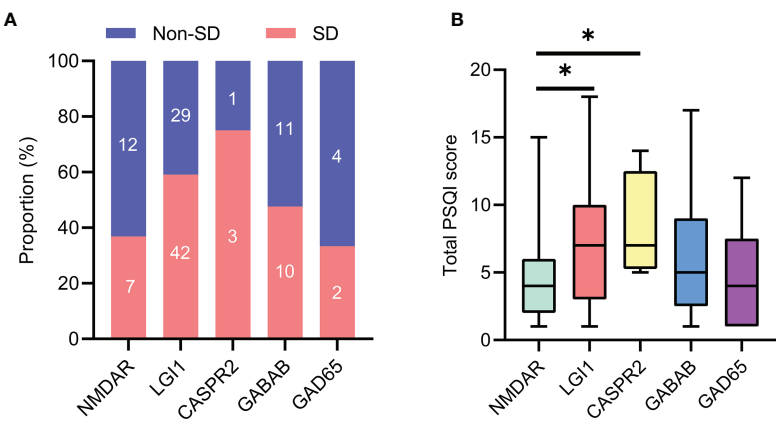

FIGURE 2 | Sleep disorders in specific antibody subtypes of AE. **(A)** Number of patients with sleep disorders in subtypes of AE. **(B)** Comparison of total PSQI score was made among subtypes of AE. AE, autoimmune encephalitis; PSQI, Pittsburgh sleep quality index. *P <0.05.

TABLE 1 | Baseline characteristics of AE patients with and without SD.

Characteristics	All patients (n = 121)	Patients with SD (n = 64)	Patients withoutSD (n = 57)	*p*-value
Age, median (IQR), years	54 (37–64)	55.4 (44.3–67.0)	49.0 (31.5–60.0)	0.001
Male, n (%)	77 (63.6)	45 (70.3)	32 (56.1)	0.106
Body mass index, mean (standard deviation)	24.5 (3.9)	24.4 (3.6)	24.5 (4.2)	0.877
Medical history, n (%)				
Hypertension	31 (25.6)	22 (34.4)	9 (15.8)	0.019
Diabetes	11 (9.1)	6 (9.4)	5 (8.8)	0.908
Smoking	33 (27.3)	23 (35.9)	10 (17.5)	0.023
Drinking	28 (23.1)	18 (28.1)	10 (17.5)	0.168
Comorbid symptoms, n (%)				
Cognitive deficits	92 (76.0)	54 (84.4)	38 (66.7)	0.023
Seizures	114 (94.2)	61 (95.3)	53 (93.0)	0.706
Psychosis and abnormal behaviors	71 (58.7)	46 (71.9)	25 (43.9)	0.002
Movement disorders	11 (9.1)	5 (7.8)	6 (10.5)	0.604
Duration of symptoms, median (IQR), days				
Cognitive deficits	235 (133–345)	233 (147–344)	255 (127–348)	0.978
Seizures	72 (37–189)	72 (46–182)	71 (28–191)	0.672
Psychosis and abnormal behaviors	85 (62–123)	88 (61–125)	87 (66–120)	0.918
Movement disorders	115 (63–284)	70 (46–343)	159 (55–294)	0.522
MoCA score, median (IQR)	20 (15–24)	17 (14–20)	24 (18–26)	<0.001
HAMA score, median (IQR)	10 (4.5–14.5)	10.5 (5.0–16.5)	10 (4.0–13.5)	0.466
HAMD score, median (IQR)	12 (6–17)	14.0 (5.3–18.0)	11 (6–16)	0.093
Tumor, n (%)	11 (9.1)	7 (10.9)	4 (7.0)	0.454
Specific antibody test, n (%)				0.288
NMDAR	19 (15.7)	7 (10.9)	12 (21.1)	
LGI1	71 (58.7)	42 (65.6)	29 (50.9)	
CASPR2	4 (3.3)	3 (4.7)	1 (1.8)	
GABAB	21 (17.4)	10 (15.6)	11 (19.3)	
GAD65	6 (5.0)	2 (3.1)	4 (7.0)	
CSF at first admission, n (%)				
CSF pleocytosis[a]	49 (40.5)	28 (43.8)	21 (36.8)	0.440
Elevated protein concentration[b]	32 (26.4)	20 (31.3)	12 (21.1)	0.204
Positive oligoclonal bands	54 (44.6)	28 (43.8)	26 (45.6)	0.837
Hyperhomocysteinemia, n (%), μmol/L[c]	41 (33.9)	26 (40.6)	15 (26.3)	0.097
NLR, median (IQR)[d]	2.6 (2.1–3.7)	2.8 (2.1–4.1)	2.4 (1.9–3.2)	0.062
NSE, median (IQR), μg/L	19.6(15.0–25.1)	21.4 (16.7–26.9)	18.5 (13.9–21.9)	0.019
Serum sodium, median (IQR), mmol/L	139 (130–141)	134 (127–140)	140 (136.5–141.5)	0.001
EEG, n (%)				0.922
Slow waves or epileptiform discharges in temporal region	78 (64.5)	41 (64.1)	37 (64.9)	
Others	43 (35.5)	23 (35.9)	20 (35.1)	
Initial MRI results, n (%)				0.130
Normal	72 (59.5)	34 (53.1)	38 (66.7)	
Medial temporal lesions	49 (40.5)	30 (46.9)	19 (33.3)	
Days from onset to diagnosis	54 (25–140)	50 (25.5–104.8)	59 (22.5–217.5)	0.584
Days from diagnosis to PET scans	6 (2–17)	6 (2–18)	5 (2–16)	0.698
Days from diagnosis to sleep assessment	9 (6–18)	9 (6–20)	9 (6–17)	0.892
Treatment, n (%)				
First-line immunotherapy				0.363
IVMP only	20 (16.5)	8 (12.5)	12 (21.1)	
IVIg only	21 (17.4)	13 (20.3)	8 (14)	
IVMP + IVIg	80 (66.1)	43 (67.2)	37 (64.9)	
Second-line Immunotherapy				
Rituximab	4 (3.3)	3 (4.7)	1 (1.8)	0.621
Mycophenolate Mofetil	8 (6.6)	5 (7.8)	3 (5.3)	0.721
Tumor removal	3 (2.5)	2 (3.1)	1 (1.8)	0.544
Tumor chemotherapy	8 (6.6)	3 (4.7)	5 (8.8)	0.473

AE, autoimmune encephalitis; SD, sleep disorders; IQR, interquartile range; MoCA, Montreal cognitive assessment; HAMA, Hamilton anxiety scale; HAMD, Hamilton depression scale; NMDAR, N-methyl-D-aspartate receptor; LGI1, leucin-rich glioma inactivated-1; CASPR2, contactin-associated protein-2; GABAB, γ-aminobutyric acid type B; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; GAD65, glutamic acid decarboxylase 65; CSF, cerebrospinal fluid; NLR, neutrophil-to-lymphocyte ratio; NSE, neuron-specific enolase; EEG, electroencephalogram; MRI, magnetic resonance imaging; PET, positron emission tomography; IVMP, IV methylprednisolone; IVIG,IV immunoglobulin.

[a]*CSF pleocytosis indicates white blood cell count of more than five cells per mm³.*

[b]*Elevated protein concentration is considered when protein levels of >45 mg/dl.*

[c]*Hyperhomocysteinemia is defined as homocysteine concentration >15 μmol/L.*

[d]*NLR is calculated by dividing the absolute neutrophil count by the absolute lymphocyte count.*

TABLE 2 | PSG findings in patients with AE compared with controls.

	Patients with AE (n = 28)	Controls (n = 18)	*p*-value
Age, median (IQR), years	57 (35–66)	55 (40–63)	0.736
Male, n (%)	19 (68)	13 (72)	0.756
Body mass index	24.2 (15.9–36.3)	24.4 (17.7–34)	0.719
Total time in bed, min	522.2 (424.4–677.3)	530.4 (420.7–598.6)	0.613
Total sleep time, min	292.8 (160–489.5)	432.0 (172–528.5)	0.001*
Sleep latency, min	37 (1.5–189)	10 (0.5–93.5)	0.005*
Sleep efficiency, %	56.1 (28–94)	80.5 (41–96)	0.001*
Wakefulness after sleep onset, min	145.8 (26.5–264.5)	76.8 (18.5–237.5)	0.004*
Stage N1, %	25.2 (4.3–56.8)	10.4 (4.7–43.2)	0.002*
Stage N2, %	51.4 (15.6–85.6)	52.4 (32.7–66)	0.893
Stage N3, %	9.4 (0–29.8)	14.3 (2.3–21.8)	0.013*
Stage REM, %	12.5 (0.7–32.5)	20.8 (13.4–32.4)	0.001*
REM sleep latency, min	139 (24–287.5)	76 (4–212.5)	0.098
Arousal index in whole sleep, n/hour	8.6 (0–33.5)	11.3 (4.2–43.6)	0.207
Arousal index in NREM sleep, n/hour	17 (0.5–33)	8.9 (3.3–18.6)	0.003*
Arousal index in REM sleep, n/hour	14 (0.9–30)	8.1 (2.5–49.6)	0.306
AHI, n/hour	10 (0–52.6)	4.3 (0–43.2)	0.140
LMI, n/hour	16 (1–360)	10 (0–32.4)	0.242
PLMI, n/hour	12 (0–25)	1.5 (0–28.3)	0.111

Results are presented as median and range.

*PSG, polysomnography; AE, autoimmune encephalitis; IQR, interquartile range; n, number; min, minutes; REM, rapid eye movement; NREM, non-rapid eye movement; N, NREM; AHI, apnea hypopnea index (n/hour); LMI, limb movements index (n/hour); PLMI, periodic limb movements index (n/hour). *p < 0.05.*

TABLE 3 | Multivariate logistic regression of independent factors associated with SD in AE.

Variables[a]	Regression coefficient	Odds ratio (95% CI)	*p*-value
Hypertension	1.081	2.946 (0.929–9.349)	0.067
Smoking	1.913	6.774 (1.238–37.082)	0.027
Drinking	−1.760	0.172 (0.026–1.160)	0.071
MoCA	−0.198	0.821 (0.752–0.896)	< 0.001
HAMD	0.072	1.074 (1.002–1.152)	0.045
Hyperhomocysteinemia	1.035	2.815 (1.057–7.496)	0.038
NSE	0.067	1.069 (1.007–1.135)	0.030

AE, autoimmune encephalitis; SD, sleep disorders; MoCA, Montreal cognitive assessment; HAMD, Hamilton depression scale; NSE, neuron-specific enolase.

[a]*These are the final variables that were retained following the application of multivariable logistic regression with backwards elimination process.*

drinking, cognitive deficits, psychosis and abnormal behaviors, MoCA score, HAMD score, hyperhomocysteinemia, NLR, NSE, serum sodium, and initial MRI. After adjusting for all included clinical variables, the logistic regression analysis showed that smoking [OR, 6.774 (95% CI, 1.238–37.082); $p = 0.027$], increased HAMD score [OR, 1.074 (95% CI, 1.002–1.152); $p = 0.045$], hyperhomocysteinemia [OR, 2.815 (95% CI, 1.057–7.496); $p = 0.038$), elevated NSE level [OR, 1.069 (95% CI, 1.007–1.135); $p = 0.03$] were independently correlated with higher risk of SD in AE patients. Contrastingly, high MoCA score [OR, 0.821 (95% CI, 0.752–0.896); $p < 0.001$] was associated with lower risk of SD in AE subjects.

Voxel-Wise Analysis of ^{18}F-FDG-PET

Figure 3 shows the significant metabolic differences of group comparisons based on voxel-based analysis between patients with and without SD, no significant difference was noted between the two groups with regard to the median time from diagnosis to the initial scan [6 (IQR, 2–18) days vs. 5 (IQR, 2–16) days, $p = 0.698$]. Patients with SD demonstrated relatively increased metabolism in the MTL, BG (medial globus pallidus, putamen, and caudate), extending to the cerebellum (cerebellum anterior lobe, cerebellum posterior lobe, dentate, cerebellar tonsil, and culmen), brainstem (midbrain and pons), thalamus, and hypothalamus compared to patients without SD ($p < 0.05$, FDR corrected; **Figure 3** and **Table 4**). Furthermore, patients with SD also showed hypometabolism in the frontal lobe (MFG and SFG) and PCC ($p < 0.001$, uncorrected; **Figure 3** and **Table 4**). Cluster extent, peak MNI coordinates, corrected p values, maximum Z value, corresponding brain regions, and voxel size for each region are detailly reported in **Table 4**.

Correlations Between ROI-Based FDG-PET Findings and Sleep Quality

To investigate and validate whether the above significant clusters containing different brain regions were truly related to sleep quality, we further conducted an ROI-based correlation analysis between patients with SD and without SD. The comparison of SUVR value between the two groups is shown in **Figure 4**. Compared with patients without SD, patients with SD showed relatively higher metabolism in the MTL (0.99 ± 0.17 vs. 0.93 ± 0.09, $p = 0.008$), BG (1.2 ± 0.12 vs. 1.14 ± 0.1, $p = 0.002$),

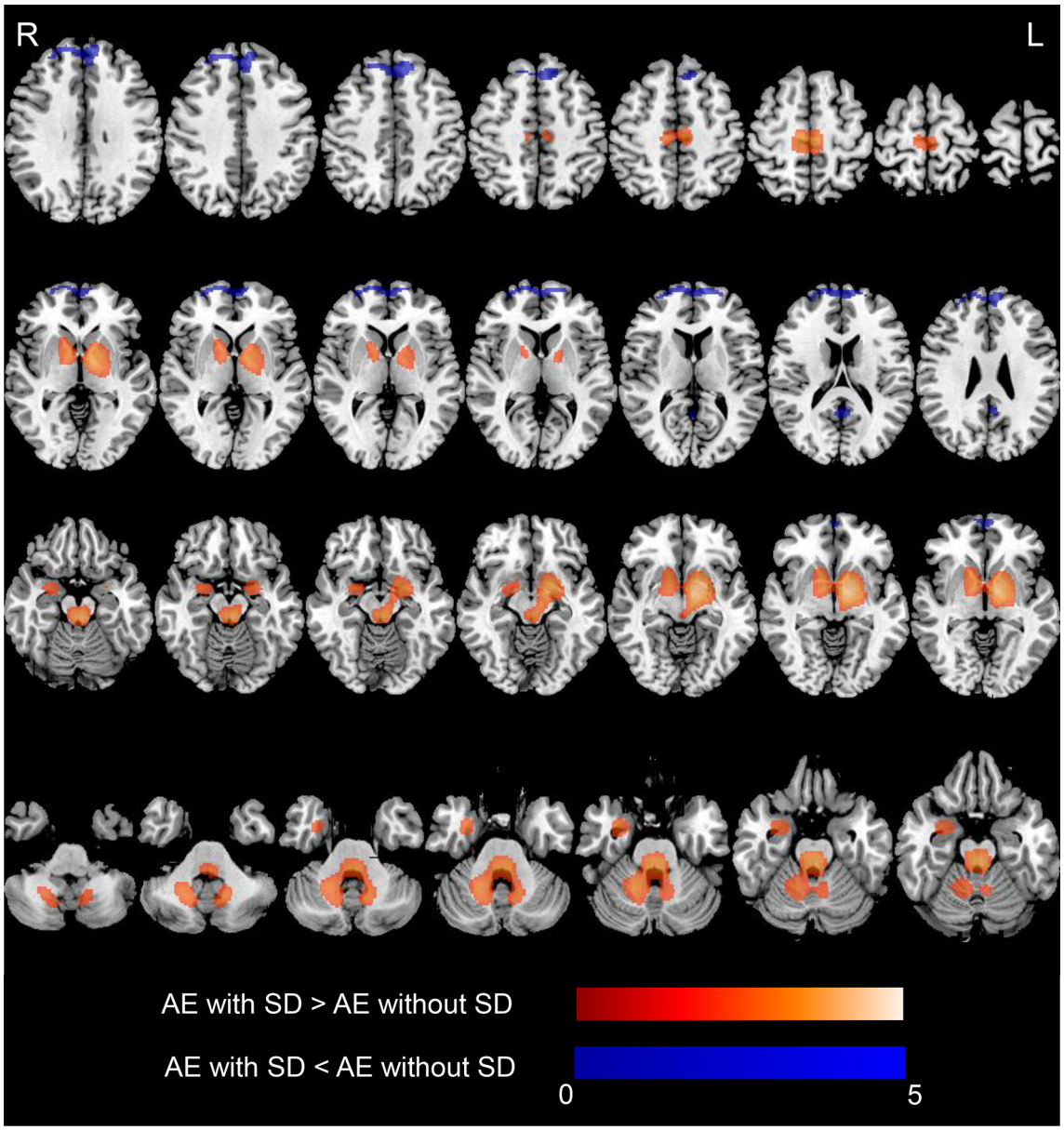

FIGURE 3 | ^{18}F-FDG-PET results based on SPM in AE patients with SD compared with those who did not develop SD. T-maps show hypo- (blue color) and hyper-metabolism (hot color). Patients with SD demonstrated relatively increased metabolism in the MTL (amygdala, hippocampus), and basal ganglia (medial globus pallidus, putamen, caudate), extending to the cerebellum (cerebellum anterior lobe, cerebellum posterior lobe, dentate, cerebellar tonsil, culmen), brainstem (midbrain, pons), and thalamus and hypothalamus ($p < 0.05$, FDR corrected). Patients with SD showed hypometabolism in the frontal lobe (medial frontal gyrus, superior frontal gyrus), posterior cingulate cortex ($p < 0.001$, uncorrected). Axial slices are shown according to radiological convention (right is left). ^{18}F-FDG-PET, ^{18}F-fluorodeoxy-glucose positron emission tomography; SPM, statistical parametric mapping; AE, autoimmune encephalitis; SD, sleep disorders; R, right; L, left.

brainstem (0.87 ± 0.07 vs. 0.83 ± 0.07, $p = 0.003$), hypothalamus (0.89 ± 0.11 vs. 0.83 ± 0.1, $p = 0.003$), CAL (1.1 ± 0.09 vs. 1.06 ± 0.09, $p = 0.033$). Patients with SD also demonstrated more increased metabolism than those without SD in thalamus (1.1 ± 0.07 vs. 1.09 ± 0.07, $p = 0.251$) and CPL (0.93 ± 0.08 vs. 0.91 ± 0.07, $p = 0.269$), but there was no significant difference between the two groups. In contrast, decreased metabolism in the SFG (0.91 ± 0.07 vs. 0.95 ± 0.06, $p = 0.001$), MFG (0.93 ± 0.08 vs. 0.98 ± 0.08, $p = 0.003$), and PCC (1.04 ± 0.12 vs. 1.1 ± 0.11, $p = 0.002$) were observed in patients with SD in comparison with those without SD.

In order to explore the relationship between PET and sleep quality in more detail, we further performed a more fine-grained PSG-based correlation analysis (**Figure 5**). Wakefulness after sleep onset was associated with abnormal metabolism in the brainstem (r = 0.52, p =0.005), SFG (r = −0.54, p =0.003), MFG

TABLE 4 | ^{18}F-FDG-PET metabolic differences of SPM comparison between AE with SD and without SD.

	Cluster extent	*p*-value	Maximum *Z* score	Peak MNI coordinates			Brain region	Voxel size
				x	y	z		
AE with SD > without SD	5514	0.038[a]	4.14	−12	−4	−6	Medial globus pallidus[c]	147
							Putamen	271
							Caudate	104
							Cerebellum anterior lobe	1341
							Cerebellum posterior lobe	421
							Dentate	337
							Cerebellar tonsil	165
							Culmen	498
							Midbrain	784
							Pons	523
							Amygdala	202
							Hippocampus	71
							Para-hippocampus gyrus	291
							Uncus	123
							Thalamus	67
							Hypothalamus	12
	807	0.038[a]	3.74	8	−24	−56	Medial frontal gyrus[c]	581
							Paracentral lobule	172
AE with SD < without SD	2012	< 0.001[b]	3.88	−6	36	46	Medial frontal gyrus[c]	871
							Superior frontal gyrus	856
							Middle frontal gyrus	93
							Orbitofrontal cortex	27
	217	< 0.001[b]	3.43	−2	−52	18	Posterior cingulate cortex [c]	191
							Precuneus	33

^{18}F-FDG-PET, ^{18}F-fluoro-2-deoxy-d-glucose positron emission tomography; SPM, statistical parametric mapping; AE, autoimmune encephalitis; SD, sleep disorders; MNI, Montreal Neurological Institute.

[a]$p < 0.05$ corrected for multiple comparisons with the false discovery rate.

[b]$p < 0.001$ uncorrected.

[c]The indicated region is the cluster's peak region.

(r = −0.51, p = 0.005), and CAL (r = 0.51, p = 0.006). Stage N1 correlated with cerebral metabolism predominantly in CAL (r = 0.62, p <0.001) and CPL (r = 0.62, p <0.001). Stage N2 was associated with metabolism in the hypothalamus (r = 0.47, p = 0.012). Stage N3 was related to glucose metabolism in PCC (r = 0.42, p =0.027), brainstem (r = −0.42, p = 0.026), MFG (r = 0.4, p = 0.033), CAL (r = −0.42, p = 0.028), CPL (r = −0.41, p = 0.029). Stage REM correlated with cerebral metabolism in MTL (r = −0.39, p = 0.041), brainstem (r = −0.28, p =0.04), thalamus (r = −0.38, p =0.049), and hypothalamus (r = -0.42, p =0.027). REM latency was associated brain metabolism in thalamus (r = -0.4, p =0.035). AI in NREM sleep was related to glucose metabolism in brainstem (r = 0.49, p =0.009), SFG (r = −0.39, p =0.042), MFG (r = −0.43, p = 0.021), CAL (r = 0.43, p =0.024). AI in REM sleep correlated with cerebral metabolism mainly in BG (r = 0.44, p = 0.021). AHI was associated with abnormal metabolism in MFG (r = −0.39, p = 0.041). There was no statistically significant correlation between other PSG variables and regional brain metabolism on FDG-PET.

DISCUSSION

In this study, we reported a cohort of 121 patients with antibody-confirmed AE and investigated the potential risk factors and metabolic mechanism for AE with SD. We found that smoking, increased HAMD score, hyperhomocysteinemia, and elevated NSE level were independently correlated with higher risk of SD. Contrastingly, high MoCA score was associated with lower risk of SD in AE subjects. In addition, compared with patients without SD, patients with SD showed increased metabolism in the basal ganglia, cerebellum, brainstem, MTL, thalamus, and hypothalamus and decreased metabolism in frontal (MFG and SFG) and PCC, suggesting that a widespread metabolic network disfunction may be involved in the pathology of SD in AE.

In recent years, many studies have shown that sleep can be evidently affected by antibody-mediated diseases. However, AE-related SDs do not attract enough attention from clinicians, and little is known about the risk factors of SD in AE. Our results demonstrated that smoking was independently associated with high risk of SD in AE. Prior study showed that high levels of tobacco exposure were associated with sleep disturbance (21), but how smoking contributes to AE-related SD remains unclear. One possible explanation was that tobacco could promote neurotransmitters disturbance (22); meanwhile, it also affected activity of B- and T-lymphocyte subsets, promoted cytokine-driven systemic inflammation, and further might act to induce autoimmune diseases (23). Therefore, when the abnormal immune reaction and neurotransmitter imbalance attack sleep-related neurons or structures may lead to sleep disorders. Understanding the

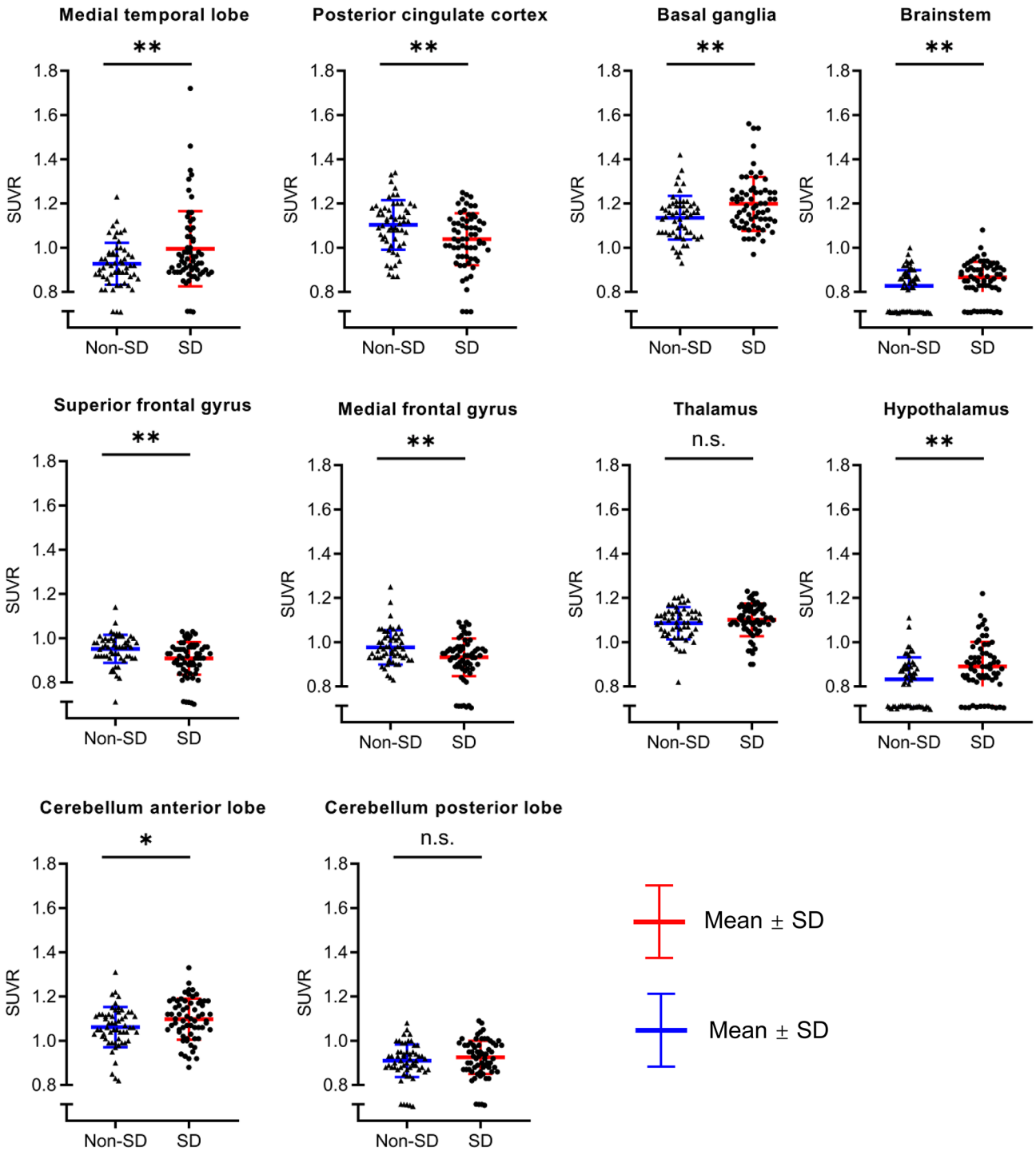

FIGURE 4 | The comparison of SUVR value between AE patients with SD and without SD. Compared with patients without SD, patient with SD showed relatively higher metabolism in the medial temporal lobe, basal ganglia, brainstem, hypothalamus, cerebellum anterior lobe. Patients with SD also demonstrated more increased metabolism than those without SD in the thalamus and cerebellum posterior lobe, but there was no significant difference between two groups. In contrast, decreased metabolism in the superior frontal gyrus, medial frontal gyrus, and posterior cingulate cortex were observed in patients with SD in comparison with those without SD. SUVR, standardized uptake value ratio; AE, autoimmune encephalitis; SD, sleep disorders; ns, no significance; *$p < 0.05$; **$p < 0.01$.

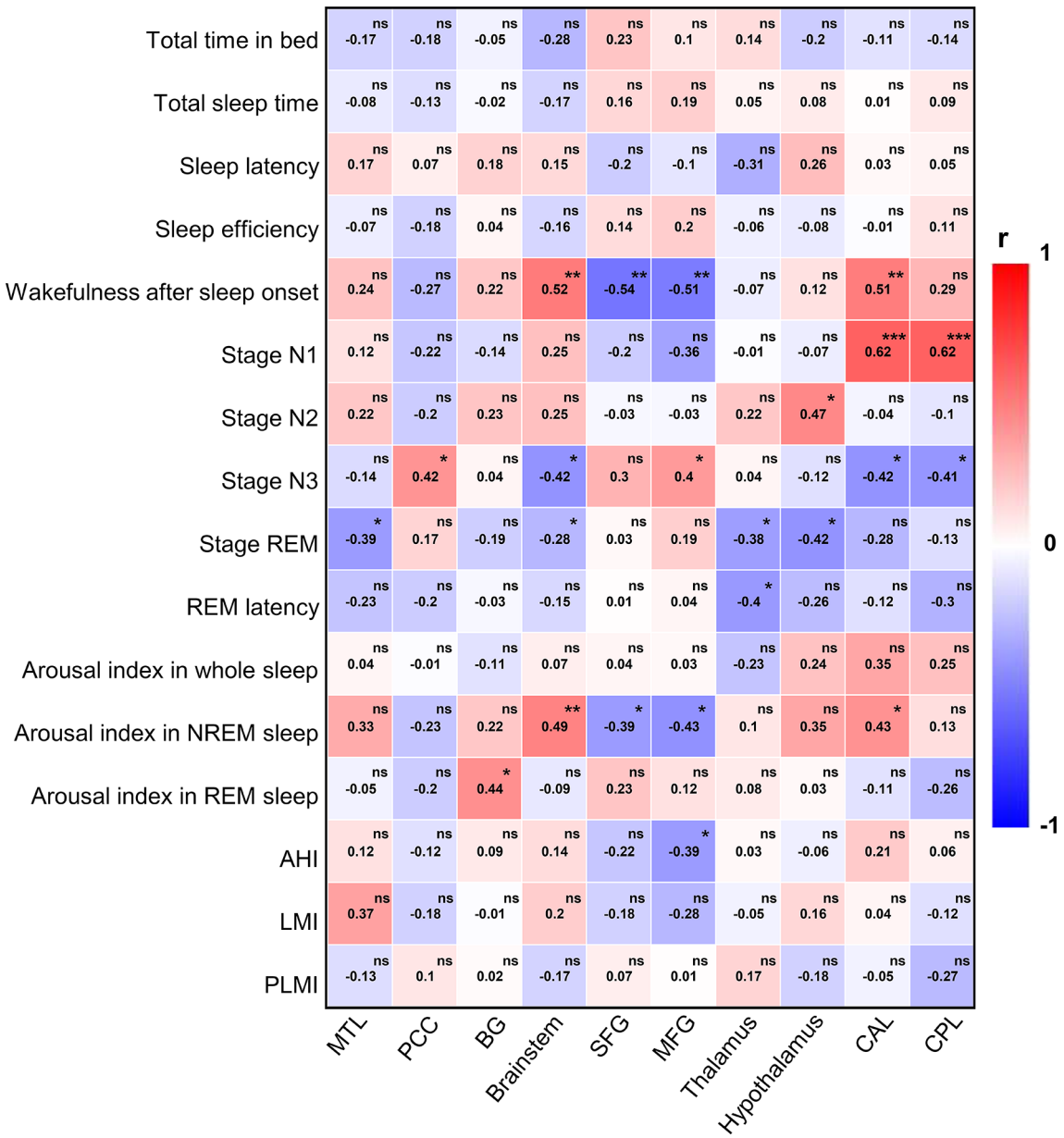

FIGURE 5 | PSG-based correlation analysis between ^{18}F-FDG-PET and sleep quality. PSG, polysomnography; ^{18}F-FDG-PET, ^{18}F-fluorodeoxy-glucose positron emission tomography; REM, rapid eye movement; NREM, non-rapid eye movement; AHI, apnea hypopnea index; LMI, limb movements index; PLMI, periodic limb movements index; MTL, medial temporal lobe; PCC, posterior cingulate cortex; BG, basal ganglia; SFG, superior frontal gyrus; MFG, medial frontal gyrus; CAL, cerebellum anterior lobe; CPL, cerebellum posterior lobe. The color bar indicates Spearman correlation coefficient and is scaled from red to blue; ns, no significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

interactions of these transmitter systems could be important for the development of SD in AE.

Hyperhomocysteinemia is an independent risk factor for SD in AE subjects in the current study. Although the potential mechanism through which elevated homocysteine acts to increase the high risk of AE-associated SD is unclear, the following reasons may explain this result. Homocysteine, as a multifunctional factor, has been generally reported to play a complex role in neurological disorders, such as stroke and neurodegenerative diseases (24, 25). Increased homocysteine mainly contributes to pathological vascular injury and aggravates inflammatory process (26); patients with OSA are frequently accompanied by hyperhomocysteinemia, which may be related to severe oxidative stress (27). However, the potential association between SD and increased homocysteine remains uncertain. We speculated that brain tissues suffered irreversible damage due to ischemia and hypoxia induced by elevated homocysteine and released a large number of harmful free radicals, which affected neural central structures and the

dopaminergic neurons regulating sleep and further caused sleep–wake dysfunction. More attention should be paid to homocysteine and its metabolic pathways in the further study.

Furthermore, we also found that the elevated NSE was correlated with increased risk of SD in AE. NSE is an important regulatory enzyme in the process of glycolysis and is widely found in neurons and neuroendocrine cells; high levels of NSE indicate the neuron injury (28). In this study, the serum NSE level in AE with SD group was higher than that in the non-SD group, suggesting that sleep disorders were closely related to neuron damage. In addition, elevated NSE was also observed in OSA patients compared to controls (29). Thus, it is proved that central nervous system injury based on immunological derangement may be an important cause of sleep disorders.

Although it has been suggested that sleep disorders are frequent and may be influenced by impaired brain regions involved in the sleep–wake regulatory network in patients with autoimmune encephalitis, the exact neuroanatomical and pathological mechanism behind sleep disfunction caused by AE is still unknown. The current findings from the aspect of brain mentalism using ^{18}F-FDG-PET indicated that, compared with patients without SD, patients with SD exhibited highly hypermetabolism in the MTL, BG, brainstem, thalamus, hypothalamus, and cerebellum and decreased metabolism in the SFG, MFG, and PCC, suggesting that AE-related SD is not confined to the limbic system but rather affects a wide range of brain regions and functional networks. Some studies showed that sleep disturbances might be related to abnormal neural network connectivity, such as the default mode network (DMN), which comprises multiple interwoven networks, and is mainly associated with memory, cognitive function, and maintenance of conscious state (30, 31). A functional MRI (fMRI) study showed that, compared with controls, patients with insomnia demonstrated increased functional connectivity (FC) between DMN and sensory-motor network and decreased FC between DMN and salience network (32). PCC and MTL, as important components of DMN, may play important roles in the regulation of sleep disorders. A prior study reported decreased ALFF values in the bilateral posterior cingulate gyrus in patients with anti-NMDAR encephalitis, which may be related to SD (33). However, functional connectivity analysis of SD in AE is lacking; future research about this should be conducted. The hypothalamus is an important sleep center involved in the regulation of sleep and wake. This may be a good explanation for SD in patients with encephalitis associated with LGI1 antibody, which is often related to hypothalamic disorders. In addition, it has been suggested that, compared with controls, AD with SD showed decreased metabolism in the hypothalamus (34). However, AE with SD exhibited hypermetabolism compared with controls, which may result from the inflammatory process in the acute stage accompanied by a rapid increase in neural antibodies and impairment of synaptic plasticity.

For PSG, AE patients had higher percent of stage N1, lower percent of stage N3, and more AI in NREM sleep than controls in the current study, which was similar to a prior study (7). Further PET-based correlation analysis showed that NREM sleep of stage N1 correlated with the cerebellum; stage N3 was related to PCC, brainstem, MFG, and cerebellum; AI in NREM sleep was related to the brainstem, SFG, MFG, and cerebellum, suggesting that cerebellum may play an important regulatory role in NREM sleep. Some studies have suggested that Purkinje cells (PCs) in the cerebellar cortex exhibited increased activity prior to the transition from sleep to wakefulness; in addition, the increased PC activity was accompanied by decreased activity in neurons of the deep cerebellar nuclei (DCN) at the NREM sleep–wakefulness transition (35). Moreover, the cerebellum might be a novel candidate for regulating sleep and/or wakefulness states *via* its interaction with arousal neurons in the ventral thalamus and hypothalamus (36). Thus, the cerebellum is at the heart of communication with arousal neurons that regulate sleep and/or arousal states.

There are several limitations in this study. (1) Due to the retrospective nature and relatively small sample size of this study, there exists a potential selection or recall bias. (2) This study measured sleep quality through subjective self-report scale; objective polysomnography can better reflect the characteristics and accurate classification of sleep disorders. However, objective results are only available in partial patients because it is often difficult to obtain, as many patients are hospitalized with psychotic or epileptic symptoms that are uncooperative with all night examination. (3) This study did not analyze the relationship between SD and different subtypes of AE due to small sample. In addition, the current study lacks multimodal imaging; in a single imaging study, it is relatively difficult to directly find the structure and function of the related changes. In the future, large-sample, homogeneous, multimodal, and cohort studies are needed to explore the pathogenesis of SD in AE.

In summary, sleep disturbances occur in more than half of autoimmune encephalitis; a significant practical implication is that testing for AE antibodies should be considered in patients who present sleep disorders and main limbic symptoms. Five risk factors of SD in AE are observed, including smoking, HAMD score, MoCA score, hyperhomocysteinemia, and elevated NSE level. It is essential to timely monitor these factors, which may help to improve diagnosis and prognosis of AE patients. Moreover, a widespread brain networks dysfunction may be the potential neuro-metabolic mechanism of SD in AE. Further larger and prospective studies are needed to clarify and validate the sleep subtypes and pathological mechanisms of SD in AE.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Beijing Tiantan Hospital that was affiliated to the Capital Medical University of the People's Republic of China. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XL and QW recruited, diagnosed, and assessed patients. XL, TY, XZ, PY, RL, CW, LA, and QW worked on the establishment of the separate databases. XL drafted a significant portion of the manuscript or figures. XZ, RL, CW, LA, and QW reanalyzed and interpreted all final data. All authors contributed to the article and approved the submitted version.

FUNDING

The study was financially supported by the National Key R&D Program of China grant (2017YFC1307500), the Capital Health Research and Development of Special grants (2016-1-2011 and 2020-1-2013), the Beijing–Tianjin–Hebei Cooperative Basic Research Program (H2018206435), the Beijing Natural Science Foundation (Z200024, 7192054), the National Natural Science Foundation of China (81771143 and 2018YFC1315201), the Application Research of Capital Clinical Characteristics (No. Z181100001718082), and the Beijing Dongcheng District Outstanding Talent Funding Project (No. 2019DCT-M-18).

ACKNOWLEDGMENTS

The authors thank the following colleagues who contributed to this study providing FDG-PET data: Chengxu Jiang and Wei Zhang.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.738097/full#supplementary-material

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frontiers in Neurology

REVIEW
published: 17 January 2022
doi: 10.3389/fneur.2021.744653

Paraneoplastic and Other Autoimmune Encephalitides: Antineuronal Antibodies, T Lymphocytes, and Questions of Pathogenesis

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

Received: 20 July 2021
Accepted: 26 October 2021
Published: 17 January 2022

Citation:
Greenlee JE, Carlson NG, Abbatemarco JR, Herdlevær I, Clardy SL and Vedeler CA (2022) Paraneoplastic and Other Autoimmune Encephalitides: Antineuronal Antibodies, T Lymphocytes, and Questions of Pathogenesis. Front. Neurol. 12:744653. doi: 10.3389/fneur.2021.744653

Autoimmune and paraneoplastic encephalitides represent an increasingly recognized cause of devastating human illness as well as an emerging area of neurological injury associated with immune checkpoint inhibitors. Two groups of antibodies have been detected in affected patients. Antibodies in the first group are directed against neuronal cell surface membrane proteins and are exemplified by antibodies directed against the N-methyl-D-aspartate receptor (anti-NMDAR), found in patients with autoimmune encephalitis, and antibodies directed against the leucine-rich glioma-inactivated 1 protein (anti-LGI1), associated with faciobrachial dystonic seizures and limbic encephalitis. Antibodies in this group produce non-lethal neuronal dysfunction, and their associated conditions often respond to treatment. Antibodies in the second group, as exemplified by anti-Yo antibody, found in patients with rapidly progressive cerebellar syndrome, and anti-Hu antibody, associated with encephalomyelitis, react with intracellular neuronal antigens. These antibodies are characteristically found in patients with underlying malignancy, and neurological impairment is the result of neuronal death. Within the last few years, major advances have been made in understanding the pathogenesis of neurological disorders associated with antibodies against neuronal cell surface antigens. In contrast, the events that lead to neuronal death in conditions associated with antibodies directed against intracellular antigens, such as anti-Yo and anti-Hu, remain poorly understood, and the respective roles of antibodies and T lymphocytes in causing neuronal injury have not been defined in an animal model. In this review, we discuss current knowledge of these two groups of antibodies in terms of their discovery, how they arise, the interaction of both types of antibodies with their molecular targets, and the attempts that have been made to reproduce human neuronal injury in tissue culture

models and experimental animals. We then discuss the emerging area of autoimmune neuronal injury associated with immune checkpoint inhibitors and the implications of current research for the treatment of affected patients.

Keywords: autoimmune neurology, autoimmune encephalitis, paraneoplastic neurological syndromes, tissue culture, animal models, immune checkpoint inhibitors, treatment

INTRODUCTION

Autoimmune encephalitides represent a rapidly expanding—and increasingly important—group of disorders characterized by the presence of an immunoglobulin G (IgG) antibody response directed against neuronal proteins. Although these antibodies were initially identified in patients with paraneoplastic (non-metastatic) neurological complications of underlying systemic cancer, autoimmune encephalitides are significantly more common in patients without neoplasia (1, 2) and are also recognized in patients treated with immune checkpoint inhibitors.

Antibodies associated with autoimmune encephalitides fall into two groups that are divided according to antigenic target. The first group, frequently paraneoplastic, is directed against neuronal proteins located in the cytoplasm and/or nuclei. The second, and much larger, group is directed against receptors or other proteins located on the neuronal cell surface membrane. Affected patients in these two groups can differ by the presence or absence of underlying neoplasia, the mechanisms of neuronal injury, and their response to treatment (3–5). Although major advances have been made in understanding the pathogenesis of conditions associated with antibodies against neuronal cell surface membrane antigens, the pathogenesis of syndromes associated with antibodies to intracellular neuronal proteins remains poorly understood. In this review, we will contrast antibodies to cell surface membrane antigens to antibodies directed against intracellular neuronal antigens, in terms of their discovery, how these antibodies may arise, and the roles of antibody and T cell-mediated immune response in producing neuronal injury. We will then discuss the emerging area of syndromes of autoimmune neuronal injury associated with immune checkpoint inhibitors and the implications current research may have on the treatment of affected patients. Conditions associated with antibody responses to non-neuronal CNS antigens, such as anti-glial fibrillary acidic protein (GFAP), anti-aquaporin 4 (Aqp4), or myelin-associated glycoprotein (MOG) or to antigens unrelated to the nervous system are outside the scope of this review.

PARANEOPLASTIC NEUROLOGICAL DISEASE AND THE DISCOVERY OF ANTINEURONAL ANTIBODIES

The concept that patients with cancer could develop syndromes of neurological injury in the absence of tumor metastasis or direct spread—conditions that are now termed "paraneoplastic neurological syndromes"—was introduced by Oppenheim in 1888, who reported the occurrence of central nervous system symptoms in a patient with uterine cancer in whom no evidence of tumor metastasis in the brain could be found at autopsy (6, 7). Around the same time, in 1890, M. Auché described peripheral nervous system symptoms in cancer patients (8). Recognition that these disorders constitute a novel area of neurological disease and their categorization into specific neurological syndromes such as subacute cerebellar degeneration [previously also termed cortical cerebellar degeneration and recently renamed "rapidly progressive cerebellar syndrome" (9)], limbic encephalitis, and sensory neuronopathy came through the work of multiple individuals over the next 70 years (10, 11) (**Table 1**). Demonstration that these disorders might be accompanied by an autoantibody response was first made by Wilkinson and Zeromski, who identified antibodies binding to neuronal cytoplasm and nuclei in patients with cancer and sensory neuronopathy (12), and subsequently by Trotter et al., who found antibodies to cerebellar Purkinje cells in a patient with Hodgkin's disease and cerebellar ataxia (13). Definitive association of an antineuronal antibody response in paraneoplastic neurological

TABLE 1 | Paraneoplastic neurological disorders[a].

Syndromes affecting the central nervous system
Cortical cerebellar degeneration (Rapidly progressive cerebellar syndrome)[b]
Encephalomyelitis
Limbic encephalitis
Bulbar encephalitis
Cerebellar degeneration (encephalitis)[b]
Myelitis
Intractable status epilepticus
Opsoclonus/ataxia
Paraneoplastic stiff-person syndrome
Syndromes affecting ganglionic neurons
Dorsal sensory neuronopathy
Autonomic neuronopathy (manifested as orthostatic hypotension, gastroparesis, etc.)
Syndromes affecting the myoneural junction
Lambert-Eaton myasthenic syndrome in the setting of small cell cancer
Myasthenia gravis in the setting of thymoma
Syndromes affecting peripheral nerves
Sensorimotor neuropathy
Axonal neuropathy
Mononeuritis multiplex: paraneoplastic vasculitis of peripheral nerves

[a] *Reprinted from Greenlee, Current Treatment Options in Neurology, 2010 (3).*
[b] *Graus et al., In the most recent updated criteria for paraneoplastic neurologic syndromes have renamed cerebellar degeneration as "rapidly progressive cerebellar syndrome (9)".*

disease began with a report by Greenlee and Brashear in 1983; they identified the antibody now known as "anti-Yo" ("PCA1") in patients with cerebellar degeneration in the setting of ovarian cancer (14) and with subsequent confirmatory work by Jaeckle et al. in 1985 (15). Soon thereafter Graus et al. and Greenlee and Lipton identified what is now known as anti-Hu ("ANNA-1") antibody in patients with sensory neuronopathy and cerebellar degeneration, respectively (16, 17). Over the ensuing years, multiple additional autoantibodies have been identified in patients with paraneoplastic neurological disease: almost all of these antibodies are directed against intracellular neuronal antigens.

The discovery of a second group of antineuronal autoantibodies, reactive against neuronal surface proteins, opened up a window into an entirely new category of neurological disease. Early work identified antibodies directed against the metabotropic glutamate receptor, mGluR1, in rare patients with Hodgkin's disease and cerebellar ataxia (18) and of antibodies directed against mGluR5 in patients with limbic encephalitis associated with Hodgkin's disease (19). Major advances, however, came through three important discoveries: (1) the detection of antibodies against components of the voltage-gated potassium channel (VGKC) complex in patients with Morvan's syndrome (20); (2) the subsequent association of this group of antibodies with limbic encephalitis and the syndrome of faciobrachial dystonic seizures (21–23); and (3) the identification of antibodies to the N-methyl-D-aspartate receptor (NMDAR) in young women with ovarian teratomas and encephalitis, with the subsequent recognition that an identical neurological syndrome associated with anti-NMDAR antibodies could occur in the absence of neoplasia (24, 25). Subsequent studies of patients with Morvan's syndrome or with faciobrachial dystonic seizures showed that the antibodies in both conditions are not directed against VGKC *per se* but rather to the adjacent surface membrane protein, contactin-associated protein-like 2 (Caspr2), and the channel accessory protein, LGI1, respectively (26). Many additional autoantibodies directed against neuronal membrane antigens have since been associated with neurological disease. It is now recognized that the overall burden of neurological disease associated with these antibodies not only outweighs that associated with antibodies directed against intraneuronal proteins but is also more common overall than viral encephalitides (1). Terminology for autoimmune neurological conditions associated with neoplasia has recently been revised, and the risk of neoplasia for different anti-neuronal and other antibodies has been stratified (9).

TABLE 2 | Representative antibodies against synaptic or other neuronal cell surface proteins and their associated clinical syndromes.

Anti-body	Major clinical syndromes	Major associated neoplasms
AntiAMPAR	Limbic encephalitis	Small cell lung carcinoma Breast carcinoma Thymoma
Anti-Caspr2	Limbic encephalitis Morvan's syndrome	Tumor associations uncommon (Thymoma)
Anti-DPPX	Encephalopathy Myelopathy GI dysmotility	Tumor associations uncommon (Lymphoma)
Anti-DR2	Parkinsonism Encephalitis	No tumor association reported
Anti-$GABA_AR$	Encephalitis Epilepsy	Tumor association uncommon (Thymoma, Hodgkin's disease, multiple myeloma)
Anti-$GABA_BR$	Epilepsy Limbic encephalitis Opsoclonus myoclonus	Small cell lung cancer
Anti-Glycine receptor	PERM Stiff Person Spectrum Disorder	Tumor associations uncommon
Anti-IGLON5	Dementia Sleep disorder Respiratory impairment	(Thymoma)
Anti-NMDAR	Limbic encephalitis Psychosis Epilepsy Movement disorders Psychosis Catatonia	Ovarian or testicular teratoma
Anti-mGluR1	Cerebellar ataxia	Hodgkin's disease
Anti-mGluR5	Limbic encephalitis "Ophelia syndrome"	Hodgkin's disease
Anti-P/Q type VGCC	Cerebellar ataxia (Lambert-Eaton myasthenic syndrome)	Small cell lung cancer

AMPAR, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid receptor; Caspr2, contactin-associated protein-like 2; DPPX, dipeptidyl-peptidase-like protein 6 encephalitis; D2R, dopamine 2 receptor; GABA, γ-aminobutyric acid; LGI1, Leucine-rich glioma-inactivated NMDA-R, anti-N-methyl D-aspartate receptor encephalitis; mGluR1, metabotropic glutamate receptor 1; VGCC, voltage-gated calcium channel.

ANTIBODIES TO NEURONAL SURFACE MEMBRANE PROTEINS

Antineuronal antibodies against neuronal membrane proteins represent the most commonly detected antibodies associated with autoimmune encephalitis, with a steadily growing list of over 50 different autoantibodies related to human neurological disease (**Table 2**). Virtually all of these antibodies are directed against known membrane proteins and may target neurotransmitter receptors. These include NMDAR, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid receptors (AMPAR), γ-aminobutyric acid $(GABA)_AR$ or $GABA_BR$, metabotropic glutamate receptors, ion channel complexes including P/Q voltage-gated calcium channels, as well as other membrane sites associated with neuronal growth or differentiation, such as immunoglobulin-like cell adhesion molecule (IGLON5). Although the syndromes associated with many of these antibodies are often categorized as "limbic encephalitis," their semiology is more complex and may involve not only hippocampus and associated limbic structures, but also the brainstem, cerebellum, and, less frequently, spinal cord. The most common of these antibodies is anti-NMDAR. In the California Encephalitis Project, anti-NMDAR encephalitis was identified

over four times more frequently than encephalitides due to herpes simplex type 1 virus, varicella-zoster virus, or West Nile virus (2, 27).

ANTIBODIES TO INTRACELLULAR NEURONAL PROTEINS

Antibodies against intracellular neuronal proteins and their associated diseases are significantly less common than those associated with antibodies directed against cell surface membrane antigens (**Table 3**), and their role in disease pathogenesis, vs. that of T lymphocytes, is not known. Some, but not all, of these antibodies are directed against known intracellular proteins: these include anti-GAD65, anti-amphiphysin, and antibodies to collapsin response-mediator protein (CRMP5). Other antibodies, however, such as anti-Yo, anti-Hu, anti-Ri, and antibodies of the anti-Ma group, target intracellular antigens whose specific biological functions have not been fully elucidated. Anti-Yo antibodies have been reported to bind to the rough endoplasmic reticulum and Golgi apparatus within Purkinje cells (28, 29), and cloning of the Yo antigen has identified two closely related proteins, a 62 kDa protein, CDR2 (30), and a closely related 53 kDa protein, CDR2L (31). Both proteins contain a leucine zipper motif. However, antibodies to CDR2L more closely duplicate the antibody binding characteristics of native anti-Yo antibodies. In addition, CDR2L reacts with ribosomal proteins, similar to the ultrastructural localization of the Yo antigen seen with intact human anti-Yo antibodies, whereas CDR2 labels nuclear speckle proteins (28, 29, 31, 32). Studies by de Graaff et al. using mass spectrometry and transfected HLA cells, indicated that 12/12 anti-Tr sera, associated with cerebellar degeneration in the setting of Hodgkin's disease, reacted with glycosylated forms of the transmembrane Delta/Notch-like epidermal growth factor-related receptor (DNER) (33). Earlier studies by Graus et al., using confocal and immune electron microscopy, detected anti-Tr immunolabelling of Purkinje cell cytosol, the endoplasmic reticulum, dendrites, and the outer surface of the endoplasmic reticulum of neurons in the molecular layer, consistent with the transmembrane/intracellular distribution of DNER in Purkinje and related neurons (34).

Antigens recognized by anti-Hu and anti-Ri antibodies are directed against RNA processing proteins encoded by the Hu and Nova family of genes, respectively (35–37). Both gene families have been associated with diverse biological functions. Anti-Hu antibody recognizes a family of genes, HuA, HuB, HuC, and HuD, which have been shown to play roles in RNA alternative splicing and polyadenylation; mRNA stability; shuttling of target mRNAs into the cytoplasm; and regulation of both localization and translation of transcripts within the cell cytoplasm and possibly also within neurites. Very little is known, however, about the actual alterations in neuronal function, which might occur following disruption of Hu protein function. One of the few studies addressing this question (35) demonstrated that Hu proteins regulate largely independent gene networks through control of overall transcript levels and alternative splicing. Importantly, these networks, despite their intrinsic diversity, intersect in controlling the synthesis of the major excitatory neurotransmitter, glutamate, and glutamate levels are severely compromised in Hu knockout mice (35). Given the importance of altered glutamate homeostasis in excitotoxicity, these data could provide a possible mechanism for anti-Hu-associated neuronal death.

TABLE 3 | Representative antibodies against intracellular neuronal proteins and their associated clinical syndromes.

Antibody	Major central nervous system syndromes	Major associated neoplasms
Antibodies reacting with cytoplasmic and/or nuclear antigens		
Anti-Yo (PCA1)	Subacute cerebellar degeneration [*Recently renamed "Rapidly progressive cerebellar syndrome"* (9)]	Carcinoma of the ovary, uterus, or fallopian tube; carcinoma of the breast
Anti-Hu (ANNA1)	Encephalomyelitis Subacute cerebellar degeneration Sensory neuronopathy Autonomic failure	Small cell lung carcinoma (Myxoid chondrosarcoma) (Merkel cell and other neuroendocrine tumors)
Anti-Ri (ANNA2)	Opsoclonus-ataxia syndrome Cerebella ataxia Encephalomyelitis	Breast carcinoma Small cell lung cancer
Anti-ANNA3	Limbic encephalitis Encephalomyelitis Progressive cerebellar syndrome	Small cell lung cancer
Anti-CRMP5	Encephalomyelitis Progressive cerebellar syndrome Chorea	Small cell lung cancer Non-small cell lung cancer Thymoma
Anti-Kelch-like protein 11	Brainstem and cerebellar syndromes Cerebellar ataxia	Ovarian, testicular, or other teratomas Seminomas
Anti-Ma 1 & 2	Limbic encephalitis, Brainstem encephalitis Progressive cerebellar syndrome	Ma1: Small cell lung carcinoma Ma2: testicular seminoma
Anti-SOX1	Progressive cerebellar syndrome	Small cell lung cancer (Non-small cell lung cancer)
Anti-Tr[a]	Subacute cerebellar degeneration	Hodgkin's disease
Antibodies reactive with intracellular synaptic or other membrane antigens		
Anti-Amphiphysin	Stiff person syndrome Limbic encephalitis	Breast cancer Small cell lung cancer
Anti-GAD65	Stiff Person Spectrum Disorder Limbic Encephalitis Cerebellar ataxia	Tumor association rare (Multiple tumor types reported in individual patients: breast, lung, thymoma, other)

ANNA, antineuronal nuclear antibody; SCLC, small cell lung carcinoma; NSCLC, non-small-cell lung cancer; CRMP-5, collapsin response mediator protein 5; GAD, Glutamic acid decarboxylase; KLHL11, Kelch-like protein-11; PCA, Purkinje cell cytoplasmic antigen; DNER, delta/notch-like epidermal growth factor-related receptor.

[a] *Anti-Tr has been shown to react with glycosylated forms of the transmembrane delta/notch-like epidermal growth factor-related receptor (DNER). This protein is expressed intracellularly as well as at the neuronal cell membrane, and studies employing confocal and immune electron microscopy demonstrated anti-Tr immunolabelling of Purkinje cell cytosol, endoplasmic reticulum, dendrites as well as outer surface of the endoplasmic reticulum of neurons in the molecular layer (33, 34).*

In contrast to Hu proteins, expression of the Nova1 and Nova2 proteins recognized by anti-Ri antibodies is tightly restricted to post-mitotic neurons in the CNS, with the expression of Nova1 occurring predominantly in the brainstem and ventral spinal cord and Nova2 predominantly within the neocortex (38). Both proteins bind to RNA in a sequence-specific manner and regulate alternative splicing *in vitro,* and both appear to be involved in the maintenance of neuronal excitatory and inhibitory homeostasis (38, 39). Microarray analyses and work with Nova -/-knockout mice have demonstrated that the most Nova-regulated exons are located in genes encoding proteins with important synaptic functions: these may include N-type and P-type Ca_v2 calcium channels as well as gephyrin, a protein that clusters inhibitory gamma-aminobutyric acid and glycine receptors (38–40).

As is the case with Hu proteins, however, no studies have addressed how the interaction of anti-Ri antibodies with their target antigens might alter RNA processing, nor have studies yet identified the downstream changes in RNA metabolism or protein encoding that might cause neuronal dysfunction or death. Beyond anti-Yo and anti-Hu, there are numerous other antibodies targeting intracellular proteins whose effects on neurons remain to be characterized, including the Ma antigens—recognized by anti-Ma and anti-Ta antibodies—that represent a family of proteins expressed in CNS neurons and in testis (41).

TABLE 4 | Risk of underlying neoplasia associated with detection of major antineuronal antibodies[a,b].

High risk (> 70% association with cancer)	
Hu	Ri
CV2/CRMP5	Yo
SOX1	Ma2/Ma
PCA2 (MAP18)	Tr
Amphiphysin	KLHL 11
Medium risk (30–70% association with cancer)	
AMPAR (>50%)	P/Q VGCC (50/90%[c])
$GABA_BR$ (>50%)	CASPR2 (50%)
mGluR5 (∼50%)	NMDAR (38%)
Low Risk (<30% association with cancer)	
mGluR1 (30%)	
$GABA_AR$ (<30%)	DPPX (<10%)
CASPR2 (<30%)	GlyR (<10%)
GAD65 (<15%)	
LGI1 (<10%)	

[a] *Modified from Graus et al.: Updated Diagnostic Criteria for Paraneoplastic Neurologic Syndromes, Annals of Neurology 2021 (9).*

[b] *Graus et al. also included risk of cancer associated with three antibodies to non-neuronal proteins: glial fibrillary acid protein (GFAP, AQP4, and MOG). These are not included in the table.*

[c] *Risk of associated cancer (small cell carcinoma) is 50% when the association is with LEMS but 90% if associated with rapidly progressive cerebellar syndrome.*

INITIATION OF THE IMMUNE RESPONSE

The molecular events that lead to antineuronal antibody-associated neurological disease in patients without underlying neoplasia have not yet been identified. An exception to this is the occurrence of anti-NMDAR encephalitis as a late complication of herpes simplex encephalitis (42). The pathogenesis of this association has not been elucidated, but congenital deficiency of Toll-like receptor 3, a key protective factor against viral encephalitis, has been reported in patients in whom herpes simplex encephalitis was followed by anti-NMDAR encephalitis (43, 44).

In contrast, classical paraneoplastic syndromes, such as those associated with anti-Yo or anti-Hu antibodies, are unique among other types of systemic autoimmune disorders in that the antigenic stimuli that initiate the immune response have been identified and shown to be elicited by antigens expressed in patient tumors. The same is true for cases of anti-NMDAR encephalitis associated with ovarian teratomas. Expression of Yo antigen(s) in tumors found in patients with paraneoplastic cerebellar degeneration is well-documented, as is similar tumor expression of neuronal antigens in tumors of patients with anti-Hu, anti-Ri, anti-Ma2, and anti-NMDAR, and $GABA_B$ antibody-associated encephalitides (41, 45–53). Similarly, $GABA_B$ expression has been detected in thymoma biopsies in the setting of $GABA_B$ encephalitis.

An important question has been whether expression of neuronal antigens is confined to the subset of tumors found in patients who experience paraneoplastic neuronal injury or whether these antigens are more commonly expressed by tumors but do not always cause neurologic injury, suggesting that additional host genetic or other factors may be involved in disease pathogenesis (54). Early work by Furneaux et al. suggested that anti-Yo antibodies labeled cells within ovarian carcinomas from those individuals with anti-Yo antibody-associated paraneoplastic cerebellar degeneration but did *not* label tumors from control patients with similar malignancies (45). Subsequent work, however, has demonstrated that two of the antigens detected by anti-Yo antibodies, CDR2 and CDR2L, can be detected in cancer patients both *with* and *without* neurological disease (55, 56). Similarly, although ovarian teratomas from patients both with and without NMDAR encephalitis express the GluN1 subunit of NMDAR, NMDAR encephalitis occurs in only a subset of these patients (51, 52). Hu antigens have been detected in small cell tumors from patients *with* and *without* neurological disease (47), and low titers of anti-Hu antibody have been detected in sera from neurologically asymptomatic patients with small cell tumors (57). The risk of underlying neoplasia associated with different antineuronal antibodies has been recently summarized by Graus et al. (9) (**Table 4**).

Work within the past few years has shed important light on the genomic and histopathological factors that separate patients with neoplasms who develop autoimmune neurological disease from those patients with similar neoplasms who remain neurologically unaffected. Anti-LGI1 encephalitis, although not usually a paraneoplastic condition, has been shown in genome-wide association studies (GWAS) to be highly associated with 27 single-nucleotide polymorphisms (SNPs) in the HLA-II region (58). Chefdeville et al., in studies of teratomas from patients with and without anti-NMDAR encephalitis, found that teratomas

from NMDAR patients more frequently contained neuroglial elements than did control tumors, and frequently contained robust T and B cell inflammatory infiltrates (52). A minority of tumors also contained elements resembling neuroglial tumors, a finding which is rare in ovarian tumors overall (52). Hillary et al., in a study of 43 cancer patients with cerebellar degeneration and anti-Yo antibodies described the existence of HLA allele association with anti-Yo mediated paraneoplastic cerebellar degeneration. These investigators noted that the association is complex, suggesting that multiple epitopes within Yo or other antigens may be involved (59). Vialatte de Pémille et. al. reported that CDR2L, but not CDR2, is enriched in ovarian cancers from patients with anti-Yo antibody response (60). In an important study, Small et al. examined ovarian tumors from patients exhibiting anti-Yo antibody response (anti-Yo PCD patients) as compared to antibody-negative controls. Tumors from patients with anti-Yo antibodies differed from controls in showing more abundant—and often massive—T- and B-cell infiltration. In some instances, these infiltrates were organized into tertiary lymphoid structures located near apoptotic tumor cells and harboring CDR2L protein deposits, a spatial association suggesting immune attack (54). In contrast to anti-Yo negative controls, 65% of anti-Yo PCD tumors presented one or more somatic mutations in genes encoding the Yo antigen, with a predominance of missense mutations, and 59% of anti-Yo PCD tumors showed recurrent gains of the CDR2L gene with tumor protein overexpression. In aggregate, these data were thought to indicate that genetic alterations in tumor cells could trigger immune tolerance breakdown, resulting in extensive tumor infiltration by T and B lymphocytes and initiation of autoimmune disease.

PATHOGENIC MECHANISMS IN DISEASES ASSOCIATED WITH ANTIBODIES TO NEURONAL SURFACE MEMBRANE ANTIGENS

Antibodies directed against neuronal membrane antigens play a direct role in disease pathogenesis. This has been most clearly demonstrated for antibodies directed against NMDAR and has been shown for several other anti-cell surface membrane antigens as well. Anti-NMDAR antibodies consistently target the N368/G369 region within the GluN1 subunit of NMDAR (61). *In vitro* studies of rat hippocampi demonstrate that antibody binding results in capping and cross-linking of receptor proteins, with subsequent receptor internalization and reduced presence of NMDAR clusters on the cell surface membrane (62) (**Figure 1A**). These studies parallel studies of receptor density in brains of rats infused intraventricularly with anti-NMDAR and also studies of receptor density in hippocampi obtained at autopsy from affected human patients (62). The binding of anti-NMDAR to its target receptor site did not appear to cause neuronal death, and reconstitution of synaptic density occurred when antibody titers were reduced (62). In all, these findings confirm a direct role for antibodies in the pathogenesis of anti-NMDAR encephalitis and, importantly, suggest that neuronal receptor function can recover and result in clinical improvement. Very few autopsy studies of anti-NMDAR encephalitis have been reported, and most of these have involved patients with a short duration of illness (63, 64). An as-yet unanswered question is thus whether prolonged neuronal exposure to anti-NMDAR antibodies in affected patients might eventually produce significant neuronal death or irreversible neuronal dysfunction.

Although antibodies to other neuronal surfaces and synaptic antigens have been less thoroughly studied, many produce similar effects on neurons. Anti-AMPAR antibodies have been shown to bind to the GluR1 and GluR2 subunits of AMPAR and to decrease receptor cluster density (65, 66), with alteration of inhibitory synaptic currents and vesicular c-aminobutyric acid transporter staining intensity (66). Antibodies to LGI1 bind to the ADAM23 (Disintegrin and metalloproteinase domain-containing protein 23) and ADAM22 within the trans-synaptic LGI1 complex and reduce total and synaptic levels of AMPA as well as of the voltage-gated potassium channel Kv1.1 (67). Antibodies to $GABA_A$ receptors similarly cause a reduction in synaptic receptor clusters (68). In contrast, antibodies to Caspr2 interrupt the interaction between Caspr2 and contactin-2 without reducing membrane receptor density (69), and anti-Caspr2 antibodies, unlike many of the other antibodies to receptor proteins are of IgG subclass 4 (IgG4) (69). Although most autoantibodies directed against neuronal membrane antigens appear to affect only the function of the target antigen, anti-IGLON5 antibodies, often clinically associated with a neurodegenerative syndrome, decrease receptor cluster density followed by disorganization of the neuronal cytoskeleton (70). Similarly, antibodies to voltage-gated calcium channels, in addition to their effect on receptor *function*, can be internalized, resulting in neuronal death (71). Antibodies to amphiphysin, although a submembrane protein, behave much like antibodies to neuronal surface membrane antigens. Sommer et al., employing anti-amphiphysin antibodies, were successful in demonstrating not only neuronal antibody uptake but also dose-dependent stiffness and spasms mimicking human stiff person syndrome (72). Antibodies to $GABA_A$ receptors similarly cause a reduction in synaptic receptor clusters (68).

PATHOGENIC MECHANISMS IN DISEASES ASSOCIATED WITH ANTIBODIES TO INTRACELLULAR NEURONAL ANTIGENS

The discovery of paraneoplastic autoantibodies led to multiple attempts to produce an animal model of antibody-mediated paraneoplastic neurological injury using passive transfer of antibodies, immunization with recombinant antigen or relevant DNA sequences, or adoptive transfer of T lymphocytes (**Table 5**) (74–76, 78, 80). Although some of these studies documented neuronal antibody uptake, none have produced the neurological findings or the extensive neuronal destruction seen in human disease (**Table 5**). Based on the failure to produce neurological disease using antibodies, it has become widely thought that paraneoplastic neurological syndromes associated with antibodies such as anti-Yo or anti-Hu cannot be antibody-mediated and hence must be T-cell-mediated. In this

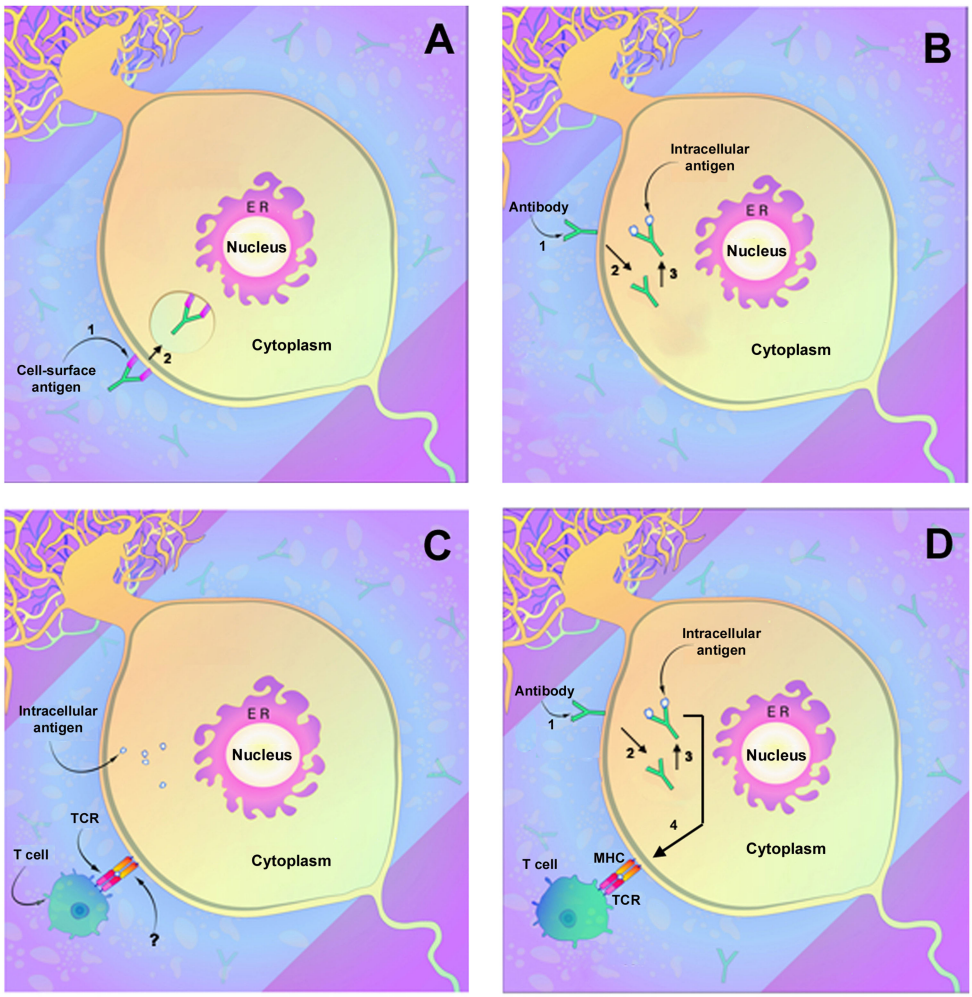

FIGURE 1 | Demonstrated and potential mechanisms of autoimmune neuronal injury. **(A)** Immune attack directed against neuronal surface membrane antigens as has been shown to occur with antibodies such as anti-NMDAR. In this instance, the antibody can decrease receptor function through either (1) binding and inhibiting the receptor or (2) by cross-linking the receptors, which facilitate internalization of receptors and reduction in membrane receptor density. **(B)** Antibody uptake and antibody-mediated neuronal injury by antibodies directed against intracellular neuronal antigens such as anti-Yo or anti-Hu. (1) Antibody attaches to the neuronal membrane, possibly by Fc-related binding, and (2) is internalized. (3) Antibody binding to its intracellular target antigen results in neuronal injury or death. **(C)** Neuronal injury by T lymphocytes. Lymphocyte T cell receptors (TCRs) interact with target neurons and cause neuronal injury or death. An area of uncertainty is that mature neurons (as opposed to fetal neurons) do not express the MHC receptors normally required for T cell interaction, and the actual mechanism of neuronal recognition by T cells is undefined. **(D)** Possible two-step mechanism of immune attack directed against intracellular neuronal antigens. As in **(B)**, the antibody binds to the neuronal membrane (1) followed by internalization (2) and binding to target antigens with resultant neuronal injury (3). Injured neurons upregulate MHC receptors (4) allowing recognition by cytotoxic T lymphocytes that also contribute to cell death. [Modified from Herdlevaer et al. (32)].

concept, antibodies themselves are simply markers of underlying malignancy. Studies to induce neurological disease using T lymphocytes, which have also been unsuccessful, have received little attention (76, 83).

Current Knowledge of the Role of T Lymphocytes in Disease Pathogenesis

The presence of cytotoxic T lymphocytes in brains and CSF of patients with classical paraneoplastic neurological syndromes associated with anti-Yo, anti-Hu, and anti-Ma2 antibodies has been extensively documented (85–89). Cytotoxic ($CD8^+$) lymphocytes have been demonstrated in the CSF of a patient with cerebellar degeneration and anti-Yo antibody response (90), and T cell clones recognizing the same antigen in brain and tumor tissue have been detected in the CSF of a patient with encephalomyelitis and anti-Hu antibodies (91). *However, not all patients with antibodies to intracellular neuronal antigens have evidence of an antigen-specific T cell response*: lymphocytic infiltrates have been absent in the brains of some patients with anti-Yo associated paraneoplastic cerebellar degeneration (14, 92); and some investigators have failed to detect cytotoxic T lymphocytes in serum or CSF of patients with anti-Hu antibodies

and paraneoplastic neurological disease (93–95). An important, but unaddressed issue concerns the ability of cytotoxic T lymphocytes to target neurons since adult neurons lack the MHC class I or class II receptors normally required for recognition by T cells (**Figure 1C**). Recent studies by Yshii et al., however, documented upregulation of MHC class 1 molecule expression in Purkinje cells in an experimental model of paraneoplastic cerebellar injury following treatment with an immune checkpoint inhibitor (96), and in ongoing studies, we have observed similar neuronal upregulation of MHC class I receptors in slice cultures incubated with anti-Hu antibodies (Carlson et al., unpublished data). Taken together, these studies document the presence of autoreactive T cells in the brains of many affected patients and suggest a mechanism by which $CD8^+$ T cells could recognize affected neurons. However, despite extensive attempts, no investigator has as yet developed an animal model of paraneoplastic neurological disease using T lymphocytes (**Table 5**) (76, 83). Attempts have included work by Tanaka et al. to produce neurological disease by adoptive transfer of mononuclear cells from a patient with paraneoplastic cerebellar degeneration into SCID (severe combined immunodeficiency disease) mice, attempts by the same group using passive transfer of T lymphocytes from mice immunized with Yo protein (76), and studies by Pellkofer et al., who studied the adoptive transfer of lymphocytes from animals immunized with the Ma-associated onconeural antigen, Pnma1, wherein recipient rats developed meningoencephalitis but not actual neuronal injury (83).

Investigations Into the Role of Antibody

The role of antibodies reactive with intraneuronal antigens, such as anti-Yo or anti-Hu, in the pathogenesis of paraneoplastic neuronal injury has been a subject of controversy. In part this has been because cytotoxic T lymphocytes have been identified in the brains of affected patients, suggesting a T cell mechanism. The controversy also remains because attempts to produce disease using these antibodies in experimental animals have been unsuccessful. In addition, neurons have historically been thought to exclude IgG, and it has thus been thought that paraneoplastic antibodies such as anti-Yo or anti-Hu would be unable to enter living neurons and react with their target antigens (97, 98). Although this concept is widely stated, both *in vivo* and *in vitro* studies have demonstrated that IgG can enter neurons and that neuronal uptake of IgG can produce neuronal injury. Early work by Fabian et al. demonstrated entry of antibodies into the central nervous system in living animals (99, 100); and Griffin et al. have demonstrated neuronal uptake of IgG in mice infected with Sindbis virus (101). Graus et al. have shown Purkinje cell uptake of normal and anti-Yo IgG in guinea pigs following intraventricular infusion (73). In short-term experiments, Greenlee et al. demonstrated similar Purkinje cell uptake of anti-Yo antibodies following intraperitoneal injection of animals in the setting of blood–brain barrier disruption, and Tanaka observed similar neuronal uptake following intracranial injection (76, 77). Relevant to human disease, intraneuronal IgG has been found in autopsied brains of individuals with encephalitis associated with both anti-Hu and anti-amphiphysin antibodies (102–104).

The failure of previous attempts to produce an animal model of paraneoplastic neuronal injury could be due to a number of factors. First, no study to date has employed or generated antibodies proven to be cytotoxic to their target neurons *in vitro;* and failure to develop an animal model could thus reflect a failure to immunize animals with the correct antigen or to use the proper antibody in experiments involving passive transfer. Case in point are the attempts to produce Purkinje cell injury using the cloned Purkinje cell antigen, CDR2 as an antigen. It is now recognized that the major Yo antigen may be CDR2L rather than CDR2 and that antibodies directed against CDR2L most closely parallel the antigen-binding seen by human anti-Yo antibodies in cerebellar sections studied using immune electron microscopy as well as by immunohistochemistry and immunoprecipitation (28, 29, 31). It is thus possible that successful production of an animal model for anti-Yo antibody-associated cerebellar degeneration may require immunization with CDR2L rather than CDR2, or, given the possible roles of each antigen in Purkinje cell protein synthesis, that immunization with both proteins might be required (32). A second issue, involved in passive transfer experiments using human IgG, could be a failure of antibody-mediated pathogenicity to occur across species lines. In early work, Greenlee et al. demonstrated that there are differences in antibody reactivity of anti-Yo and anti-Hu antibodies among IgGs from different patients and also among different animal species (105). An additional challenge in developing an animal model is that detection of early, possibly widely scattered, neuronal loss could be difficult using the conventional histological methods employed in essentially all animal studies. Importantly, no study to date has used human paraneoplastic IgG to affinity purify target antigen(s) from neurons of the species to be studied and then employ these for direct immunization or to generate antibodies for passive transfer.

A final, major challenge in producing an animal model of human paraneoplastic disease using antibody has to do with achieving sustained exposure of neurons to antibodies across the blood-brain barrier, given that neurological symptoms in human cases associated with antibodies such as anti-Yo or anti-Hu are believed to be the result of *progressive* neuronal death over time. Robust neuronal uptake of IgG has been clearly demonstrated in three separate studies using direct intracranial injection, but antibody uptake has been minimal or has not occurred in longer-term immunization or passive transfer studies (73, 74, 77). As an example, Sillevis Smitt et al., in a carefully done study using passive transfer of human anti-Hu IgG, failed to show entry of IgG into brain parenchyma or neurons (78). Additional experiments employing immunization with recombinant HuD resulted in high antibody titers but, again, did not show antibody penetration across the blood-brain barrier or entry of antibodies into brain parenchyma or neurons within the timeframe of the study (78).

One approach to studying antibody-neuron interactions in the absence of a blood-brain barrier has been through the use of tissue culture systems. In older work, anti-Hu antibodies

TABLE 5 | Major experimental attempts to produce an animal model of paraneoplastic neurological disease associated with antibodies targeting intracellular neuronal antigens.

References	Antigen targeted	Species	Method	Study duration	Outcome
Graus et al. (73)	Yo	Guinea pigs	Intraventricular infusion of anti-Yo or normal IgG	15 days	Uptake of both anti-Yo and normal IgG by Purkinje cells on day 16 but not at days 22 and 45. No observed Purkinje cell death or neurological change in animals
Tanaka et al. (74)	Yo	Mice	Intracranial injection of human anti-Yo IgG with and without complement or activated monocytes	Up to 50 h	Uptake of human anti-Yo IgG by Purkinje cells. No detected Purkinje cell loss
		Mice	Immunization with recombinant Yo protein	15 days for pathology, 3 months for observation	Development of high antibody titers No definite uptake of antibody by Purkinje cell
		Rats	Intraventricular injection	1 week	No Purkinje cell loss. Brains not studied for antibody uptake by neurons.
Tanaka et al. (75)	Yo	Mice	Immunization of multiple mouse strains with recombinant Yo protein	>2 months	No Purkinje cell loss or ataxia Strong peripheral anti-Yo production Brains not studied for uptake by neurons.
Tanaka et al. (76)	Yo	Mice	Injection of human anti-Yo IgG into occipital lobes	50 h	Antibody uptake by Purkinje cells. No Purkinje cell loss
		Mice	Injection of mouse recombinant anti-Yo IgG into mouse brain parenchyma	3–4 months	High titers of anti-Yo antibody. No neurological abnormalities No Purkinje cell loss Brains not studied for antibody uptake by neurons
		Mice	Adoptive transfer) of lymphocytes from mice immunized with recombinant Yo protein with and without recombinant anti-Yo antibodies	1 month	No neurological abnormalities No Purkinje cell loss Brains not studied for antibody uptake by neurons.
		SCID Mice	Adoptive transfer of peripheral mononuclear cells from a patient with anti-Yo antibody	1 month	No neurological abnormalities No Purkinje cell loss Attempts to detect intraneuronal IgG not described
Greenlee et al. (77)	Yo	Rats	Intraperitoneal injection of human anti-Yo antibody following blood-brain barrier disruption	4 days	Anti-Yo IgG uptake by Purkinje cells. No evidence of Purkinje cell death No neurological abnormalities
Sillevis Smitt et al. (78)	Hu	Mice	Passive intravenous transfer of human anti-Hu IgG	48 h	No evidence of anti-Hu IgG in brains of animals perfused to remove intravascular IgG No evidence of antibody uptake by neurons in perfused brains [a]
		Mice, Rats, Guinea pigs	Immunization with HuD recombinant protein	Up to 21 weeks	High serum antibody titers: No evidence of penetration of IgG into brain parenchyma or neurons in brains perfused to remove intravascular IgG
Tanaka et al. (79)	Yo	Mice	Immunization of female mice with recombinant protein; evaluation of offspring to detect transplacental passage of antibody to offspring with undeveloped blood-brain barriers	At birth and later	No Purkinje cell loss at birth No ataxia in newborn animals allowed to mature Brains not studied for antibody uptake.
Sakai et al. (80)	Yo	Mice	Immunization of mice with recombinant PCD17 protein generated using anti-Yo antibody (81)	1 year	Generation of high serum antibody titers. Presence of IgG in Purkinje cells of immunized mice, No identified Purkinje cell death or neurological abnormalities
Sakai et al. (82)	Yo	Mice	Immunization with DNA encoding recombinant PCD17 protein	Up to 1 year	Generation of antibody response which could lyse syngeneic myeloma cells pulsed with H-2K-restricted PCD17 peptide. No Purkinje cell loss or neurological abnormality Brains not studied for antibody uptake.

(Continued)

TABLE 5 | Continued

References	Antigen targeted	Species	Method	Study duration	Outcome
Pellkofer et al. (83)	Ma1		Adoptive transfer of lymphocytes from syngeneic rats immunized with recombinant Ma1 protein	9 days	Meningeal and perivascular inflammatory changes. No evidence of neuronal injury
Sakai et al. (84)	Yo	Mice	Immunization with recombinant yeast expressing recombinant (pcd17) Yo antigen	6 months	Generation of antibodies reactive with Purkinje cells and of T lymphocytes sensitized to pcd17 No clinical signs or Purkinje cell loss. Brains not studied for antibody uptake.

This study contained important controls for the detection of adventitious entry of antibodies into neurons in post mortem tissue sections.

were shown to be taken up by rat cerebellar granule cells in dispersed cultures and to cause neuronal death (106). More recently, two groups of investigators have employed organotypic (slice) cultures of rodent brains to study the interaction of normal and paraneoplastic IgGs in the absence of a blood–brain barrier, using a system in which this interaction can be studied in real time. These studies have shown that, in slice culture, Purkinje and other neurons are able to take up and clear normal IgG (107). In contrast to normal IgG, internalized anti-Yo, anti-Hu, and anti-Ri IgGs *bind* to their target antigens, and accumulate intracellularly, with anti-Yo IgG concentrated predominantly in Purkinje cells and anti-Hu and anti-Ri antibodies in multiple neuronal populations (108–111) (**Figure 1B**). Antibody uptake is rapid and, in studies done in real time, can be observed within 4 h (109, 111). The effects of antibody accumulation differ among anti-Yo, anti-Hu, and anti-Ri antibodies. Anti-Yo causes cell death largely limited to Purkinje cells (111). In the anti-Yo experimental model, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stains of injured Purkinje cells are negative, suggesting that cell death is non-apoptotic (111). In contrast, incubation of cultures with anti-Hu antibodies results in neuronal death which appears to be at least in part apoptotic (109). Although anti-Ri IgGs are widely taken up by neurons, neuronal death has *not* been detected. These observations raise questions as to whether anti-Ri antibody, at least initially, may cause neuronal dysfunction rather than neuronal death, in keeping with clinical observations showing partial clinical improvement in anti-Ri patients following treatment (109, 112). The actual mechanisms involved in neuronal death for both anti-Yo and anti-Hu antibodies are incompletely understood, and although multiple biological effects of anti-Hu and anti-Ri antibodies have been postulated, none has been directly associated with neuronal death or dysfunction. Of note, however, studies by Schubert et al. and Panja et al. indicate that anti-Yo antibodies may alter neuronal mitochondrial calcium homeostasis, possibly providing a mechanism for Purkinje cell death (110, 113). Studies addressing the functional consequences of uptake of anti-Ma2, anti-Tr, or other antibodies to intracellular neuronal proteins have not been reported.

Although *in vitro* studies with anti-Yo and anti-Hu antibodies suggest that paraneoplastic autoantibodies may play a direct role in neuronal injury in the absence of T lymphocytes, these findings have not been duplicated in living animals. The possibility also exists that antibody uptake could render neurons susceptible to attack by cytotoxic T cells by upregulation of neuronal MHC class I receptors or by other mechanisms (54, 96) (**Figure 1D**). Finally, it is possible that multiple of these mechanisms of pathogenicity may be at play during the course of the disease.

AUTOIMMUNE AND PARANEOPLASTIC ENCEPHALITIDES ASSOCIATED WITH IMMUNE CHECKPOINT INHIBITORS

The advent of immune checkpoint inhibitors as treatments for advanced malignancy has been accompanied by the unintended induction of several categories of autoimmune neurological disease including meningoencephalitis, limbic encephalitis, polyradiculitis, cranial polyneuropathy, myasthenic syndrome, and myositis (114). In addition, the use of these agents has resulted in cases of disorders classically considered paraneoplastic, including sensory neuronopathy, limbic encephalitis, and cerebellar syndrome (115–120). The major antibodies detected have been anti-Hu, and anti-Ma2, although single cases have been associated with anti-Ri, anti-CRMP5, anti-PCA-2, anti-GAD65, and other antibodies (119, 121–126). To date, an association with anti-Yo antibodies has not been reported. Three cases have been described with complex antibody responses involving anti-Hu and other paraneoplastic autoantibodies including anti-CRMP5/CV2, anti-SOX-1, anti-VGKC, or anti-NMDAR (115, 127). Cases associated with anti-Hu antibody response have occurred predominantly in patients with small cell or Merkel cell tumors, or patients with myxoid chondrosarcoma, i.e., predominantly (but not universally) in tumors classically associated with anti-Hu antibodies. In contrast, the cases involving anti-Ma2 antibodies have all occurred in patients with tumors not normally associated with anti-Ma2 antibody response, including renal cell carcinoma (119, 121, 128). In some cases, patients developing paraneoplastic neurological symptoms during immune checkpoint inhibitor therapy had detectable serum titers of paraneoplastic autoantibody *before* checkpoint inhibitor treatment was begun (115, 129, 130). Some—but by no means all—of the reported cases have responded to some extent to treatment with corticosteroids or other modalities, in combination with temporary or indefinite cessation of the specific cancer-directed immunotherapy.

The mechanisms underlying autoimmune and paraneoplastic encephalitides associated with immune checkpoint inhibitors may be disinhibition of an immune response against an autoantigen shared between the tumor and neural tissue and, as noted, some treated patients have had detectable autoantibodies prior to receiving immune checkpoint inhibitors. An experimental model of this type of this disinhibition has recently been reported by Yshii et al. using a genetically modified mouse model that specifically expressed an exogenous neoantigen, hemagglutinin (HA) in Purkinje cells and transplanted tumor cells (96, 131). These mice were then challenged to mount an immune response with a transplanted tumor that also expressed HA. Mice treated with a monoclonal antibody to VLA4 (ipilimumab) to break immune tolerance exhibited neurological disease and Purkinje cell loss. In contrast, in the absence of the anti-VLA4 antibody, none of these mice exhibited any neurological disease or Purkinje cell death. Yshii et al. also detected CD8+ T cells associated with dying Purkinje cells in their animal model and, in addition, reported two human autopsy cases of paraneoplastic cerebellar degeneration in which $CD8^+$ T cells were closely associated with areas of Purkinje cell death (96).

ISSUES AND IMPLICATIONS FOR PATIENT CARE

Neurological disorders in patients with antibodies to cell surface membrane antigens, such as anti-NMDAR, involve potentially reversible neuronal receptor impairment. Successful treatment of these conditions, with marked patient improvement, has been repeatedly documented following the use of corticosteroids, plasma exchange, intravenous immunoglobulin G (IVIG), and corticosteroid-sparing immunosuppressive agents such as rituximab, cyclophosphamide, or mycophenolate mofetil (132–134). To date, no single agent or sequence of agents has been proven to be more effective, and optimal use of therapeutic agents and length of treatment await prospective controlled clinical trials. The initial response to immunotherapies can be slow, often tempting clinicians to layer multiple immunosuppressive medications without clear endpoints. Recovery is frequently prolonged and requires multidisciplinary support, especially for cognitive and psychiatric symptoms (135).

Neurological injury in patients with paraneoplastic antibodies to *intracellular* neuronal antigens, such as anti-Yo or anti-Hu, represents a different category of disease. Clinical findings in these conditions are ultimately the result of immune-mediated neuronal death, and neurological deficits develop as neurons die and neuronal reserve is exhausted. In these disorders, substantial improvement does not tend to occur, and for this reason, even more than with conditions such as anti-NMDAR encephalitis, time is of the essence in initiating treatment.

Four different considerations come into play in treating affected patients with antibodies such as anti-Yo or anti-Hu. First, early diagnosis and treatment of the underlying tumor, with the removal of the tumor as an antigenic stimulus, is widely considered to provide the greatest chance for neurological improvement or symptom stabilization (136). However, in many cases, this goal cannot be achieved: the underlying tumor may not as yet be detectable or, conversely, the tumor may be sufficiently advanced that treatment is purely palliative. In both instances, immunotherapy may be the mainstay of treatment in the face of as-yet undiagnosed or incurable malignant disease.

The second consideration in treatment has to do timing. In most series and many case reports, treatment was started after symptoms were well-advanced, often weeks or months after symptom onset. Widdess-Walsh et al., in a review of cases of paraneoplastic cerebellar degeneration treated with IVIG, found that most patients having a good response were treated within 1 month of symptom onset, whereas outcome in patients treated after 3 months when neurological deficits were more advanced, was usually limited (137, 138). This finding—that patients with severe deficits respond poorly—has been confirmed by other investigators (139). An important question in many of the reported cases is thus whether treatment was instituted too late to be of value (137–139).

The third consideration has to do with the choice of treatment regimens. Results of treatment reported in the literature have been largely disappointing, with little progress in approaches to treatment over the last 30 years (137–139). Corticosteroids, plasma exchange, immunoadsorption, IVIG, rituximab, cyclophosphamide, and agents such as sirolimus have all been used as immunotherapies (3, 140). However, there has been little uniformity of treatment, even within the given series, and the actual effect of any of these treatments on key T- or B-cell-mediated pathophysiology in paraneoplastic neurological injury remains unclear. Plasma exchange, although effective in reducing serum antibodies, may not sufficiently reduce titers of antibodies produced within the central nervous system (141). Similarly, although rituximab has a profound effect on pre-B cells and B cells, it does not affect plasma cells and in one study did not reduce serum or CSF antibody titers (142). Cyclophosphamide, despite its effects on both T and B cells, may also fail to reduce antibody titers in paraneoplastic neurological disease (143). In the absence of an animal model, the effect of any of these modalities on T-cell function remains undefined. IVIG has multiple potential effects on immune function, but we do not yet know which of these is important in preventing the progression of paraneoplastic neurological injury. Although the use of the plasma cell depleting agent, Bortezomib, has not been reported in paraneoplastic disorders associated with antibodies to *intracellular* antigens, the agent has been shown to reduce antibody titers and produce clinical improvement in NMDAR encephalitis (144).

An additional consideration has to do with the potential role of antineuronal antibodies in causing paraneoplastic neurological disease. Tissue culture studies demonstrating uptake of antibody by neurons raise the question as to whether the use of agents capable of blocking neuronal antibody uptake might represent a potential adjunctive therapeutic approach to these disorders, providing some degree of protection of neurons from antibodies already present in CSF and brain. Colchicine has been shown to prevent uptake of anti-Yo antibodies in slice cultures, presumably by its effect on microtubules (111); however,

its clinical use would be limited by its toxicity and narrow therapeutic range. Congdon et al., in studies of a mouse model of Alzheimer's disease, demonstrated that neuronal antibody uptake could be blocked by the clathrin inhibitor, chlorpromazine (145). In the case of paraneoplastic cerebellar degeneration associated with anti-Yo antibody, work by Panja et al. suggests that minimizing intracellular calcium overload toxicity either directly with cyclosporin-A or indirectly with cannabidiol or the ROS scavenger butylated hydroxytoluene could potentially provide neuroprotection by stabilizing mitochondrial calcium homeostasis (110, 113).

A final concern has to do with the way forward in treating this group of patients. The rarity of the classical paraneoplastic disorders and the small number of cases seen at any one institution make conventional multi-institutional controlled trials difficult to achieve. An alternative approach, facilitated by the rapid growth of autoimmune neurology as a specialty, could be a study involving a large number of sites in which enrollment required patients to be ambulatory and cognitively intact, and in which specific, uniformly applied treatment protocols are used. Ultimately, understanding of pathogenesis and imaginative development of therapeutic approaches to this group of disorders awaits the development of successful animal model systems in which such treatments could be tested, and their effects on T cell function and on antibody uptake by neurons and neuronal injury could be studied in detail.

AUTHOR CONTRIBUTIONS

JG, NC, SC, and CV conceived and wrote the initial draft of this manuscript. JA and IH contributed to the manuscript draft. JG, NC, SC, JA, IH, and CV completed the final revision as submitted. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by Merit Review Awards from the United States Department of Veterans Affairs to (JG and NC), awards from the Western Institute for Biomedical Research to (JG and SC), and by grants from Helse Vest to (CV).

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frontiers in Neurology

ORIGINAL RESEARCH
published: 02 March 2022
doi: 10.3389/fneur.2022.689975

Evaluation of Plasma Neurofilament Light Chain Levels as a Biomarker of Neuronal Injury in the Active and Chronic Phases of Autoimmune Neurologic Disorders

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OPEN ACCESS

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

Received: 01 April 2021
Accepted: 01 February 2022
Published: 02 March 2022

Citation:
Kammeyer R, Mizenko C, Sillau S, Richie A, Owens G, Nair KV, Alvarez E, Vollmer TL, Bennett JL and Piquet AL (2022) Evaluation of Plasma Neurofilament Light Chain Levels as a Biomarker of Neuronal Injury in the Active and Chronic Phases of Autoimmune Neurologic Disorders. Front. Neurol. 13:689975. doi: 10.3389/fneur.2022.689975

Objective: To evaluate plasma neurofilament light (NfL) levels in autoimmune neurologic disorders (AINDs) and autoimmune encephalitis (AE).

Background: Each particular neural autoantibody syndrome has a different clinical phenotype, making one unifying clinical outcome measure difficult to assess. While this is a heterogeneous group of disorders, the final common pathway is likely CNS damage and inflammation. Defining a biomarker of CNS injury that is easily obtainable through a blood sample and reflects a positive treatment response would be highly advantageous in future therapeutic trials. Measurement of blood concentration of neurofilament light (NfL) chain, however, may provide a biomarker of central nervous system (CNS) injury in AE and other AINDs. Here we provide an initial evaluation of plasma NfL levels in AE as well as other AINDs during active and chronic phases of disease and demonstrate its potential utility as a minimally-invasive biomarker for AE and AINDs.

Design/Methods: Patients were retrospectively identified who were enrolled in the biorepository at the Rocky Mountain MS Center at the University of Colorado, or were prospectively enrolled after initial presentation. Patients had a well-defined AIND and were followed between 2014 and 2021. NfL was tested using the Single Molecule Array (SIMOA) technology. Patients with headaches but without other significant neurologic disease were included as controls.

Results: Twenty-six plasma and 14 CSF samples of patients with AINDs, and 20 plasma control samples stored in the biorepository were evaluated. A positive correlation was found between plasma and CSF NfL levels for patients with an AIND ($R^2 = 0.83$, $p < 0.001$). Elevated plasma levels of NfL were seen in patients with active AE compared to controls [geometric mean (GM) 51.4 vs. 6.4 pg/ml, $p = 0.002$]. Patients with chronic symptoms (>6 months since new or worsening symptoms) of AE or cerebellar ataxia

(CA) showed a trend toward lower plasma NfL levels (GM 15.1 pg/ml) compared to active AE or CA. Six patients with longitudinal, prospective sampling available demonstrated a trend in decreased plasma NfL levels over time.

Conclusions: Our findings support the use of plasma NfL as a potential minimally-invasive biomarker of CNS injury.

Keywords: autoimmune encephalitis (AE), neurofilament (NF), biomarker, autoimmune neurological disorders, neurofilament light (NfL) chain, cerebellar ataxia

INTRODUCTION

Over the past decade, multiple autoimmune neurologic disorders (AINDs) mediated by pathogenic neuronal cell surface antibodies (neuronal surface antibody syndromes, or NSAS) have been identified. AINDs encompass all neurologic isolated inflammatory disease thought to be mediated by the adaptive immune system–including autoimmune encephalitis (AE), stiff person spectrum disorder (SPSD), autoimmune cerebellar syndromes, and demyelinating diseases like neuromyelitis optica spectrum disorder (NMOSD) or anti-myelin oligodendrocyte (MOG) antibody disease (MOGAD). Both observational and retrospective studies have reported improved clinical outcomes with immunotherapy (1–5) in AINDs; however, there remains a strong need for randomized, controlled clinical trials to establish a standard of care for the treatment of AE and the non-demyelinating AINDs. While change in seizure frequency and cognitive functional status have been used as outcome measures for AE therapy, these measures are problematic end-points due to their poor specificity and sensitivity across the heterogenous presentations of even AEs caused by the same autoantibody (6). Some AEs may cause neuronal destruction, while others may cause dysfunction only by blocking signaling, interfering with synaptic architecture, or receptor internalization. Therefore, defining a unifying, quantitative biomarker of central nervous system (CNS) injury in AEs that is readily obtainable through a blood sample would significantly advance clinical research.

Neurofilaments are neuron-specific cytoskeletal proteins that are released following axonal damage (7). Elevated levels of NfL have been interpreted as reflecting axonal damage and neuronal death in MS (7, 8), neurodegenerative dementia (9–11), and motor neuron disease (12, 13). In MS, NfL in serum highly correlates with CSF levels (14). In addition to correlating with disease activity on MRI (7), NfL serves as a promising prognostic and therapeutic biomarker in MS (14–16).

Prior studies have described CSF levels of NfL in AE (17–19), however there is little data on serum NfL levels in AE (20). One study examined CSF levels of NfL in a cohort of 25 subjects with autoimmune encephalitis (including seronegative antibody syndromes, NSAS [n =5; 4 NMDAR and 1 LGI1], and intracellular antibody syndromes) with evidence of elevated CSF NfL at the time of diagnosis correlating to disability at 1 year (18). An additional retrospective study examined progranulin (PGRN) in both serum and CSF in 38 patients with AE [NMDAR $n = 18$, Caspr2 $n = 8$, LGI1 $n = 10$, GABA-bR $n = 1$, and AMPAR $n = 1$]; CSF NfL ($n = 25$) and t-tau ($n = 13$) was also measured in these patients (17). In this cohort, 3 NMDAR patients had highly pathological CSF NfL levels that seemed to best characterize the state of neuronal death in the brain. These studies had evaluated CSF NfL using commercial enzyme-linked immunosorbent assay (ELISA; UmanDiagnostics AB, Umeå, Sweden). Another recent study of 25 patients with autoimmune encephalitis (NMDAR, $n = 10$; LGI-1, $n = 9$; Caspr2, $n = 3$; both LGI1 and Caspr2, $n = 1$, GABA-bR, $n = 1$; AMPAR, $n = 1$), demonstrated elevated serum levels of NfL that correlated with elevated levels of CSF NfL (20). This particular study, similar to our study, used a highly sensitive assay for NfL testing using the SIMOA platform.

In our study, we evaluated NfL in the plasma of 26 patients with various AINDs, along with 20 control patients looking at both active and chronic phases of each AIND. Fourteen of 26 AIND patients also had matched CSF available for NfL testing.

MATERIALS AND METHODS

Patients

Patients enrolled in the biorepository specimen bank at the University of Colorado Anschutz Medical Campus from 2014 to 2021 were identified retrospectively. Between 2019 and 2021, patients who were evaluated in the Autoimmune and Neuroimmunology/Multiple Sclerosis outpatient clinics or inpatient neurology service at the University of Colorado with a well-defined AIND were enrolled prospectively into our autoimmune, paraneoplastic and inflammatory neurological disease registry and biorepository. A fellowship-trained Neuroimmunologist made diagnosis of a well-defined AIND. Patients who had been enrolled in the biorepository specimen bank for a primary evaluation of headache without other significant neurologic symptoms were identified retrospectively to serve as a control group. All patients or legal representatives

Abbreviations: AE, Autoimmune Encephalitis; AMPAR, Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AE, Autoimmune encephalitis; AINDs, Autoimmune neurological disorders; Caspr2, Contactin-associated protein-like 2; CBA, Cell-based assay; CNS, Central nervous system; CA, Cerebellar Ataxia; CSF, Cerebrospinal fluid; DPPX, Dipeptidyl-peptidase-like protein-6; EEG, Electroencephalogram; ELISA, Enzyme-linked immunosorbent assay; GM, Geometric mean; GAD, Glutamic acid decarboxylase; GlyR, Glycine receptor; GFAP, Glial fibrillary acidic protein; GABA-aR, g-aminobutyric acid type A receptor; LGI1, Leucine-rich glioma-inactivated-1; MRI, Magnetic resonance imaging; MOG, Myelin oligodendrocyte glycoprotein; MOGAD, Myelin oligodendrocyte glycoprotein antibody disease; NfL, Neurofilament light; NMOSD, Neuromyelitis optica spectrum disorder; NSAS, Neuronal cell surface antibody syndrome; NMDAR, N-methyl-D-aspartate receptor; SPSD, Stiff person spectrum disorder; TRIM46, Tripartite Motif Containing 46.

consented to enrollment in the autoimmune, paraneoplastic and inflammatory neurological disease registry and biorepository [approved by the Colorado Multiple Institutional Review Board (COMIRB)].

We included 26 patients with CNS autoimmune neurological syndromes, with 18 of these patients having active symptoms, and 8 having chronic symptoms. Patients were defined as having active AE if they had experienced new or worsening symptoms of altered mental status, impaired cognition/memory, personality/behavioral change, seizure frequency, decreased speech or mutism, or centrally-mediated movement disorders (ataxia, chorea) in the past 6 months, while being on or off immunotherapy. Patients were defined as having active SPSD if they had experienced new or worsening symptoms of SPSD (muscle rigidity/spasms, hyperstartle) without concurrent encephalitic symptoms in the past 6 months. Patients were defined as having active autoimmune cerebellar ataxia (active CA) if they had experienced worsening of cerebellar symptoms without concurrent encephalitic symptoms in the past 6 months. Active symptoms for all patients could be either at initial disease onset or during a relapse. Patients who had experienced no recent new or worsening encephalitic or cerebellar symptoms in the 6 months prior to sample collection were defined as having chronic autoimmune encephalitis /cerebellar ataxia (chronic AE/CA). All patients were included only once in analysis based on clinical presentation at the time of initial sampling. A cut-off of 6 months for active (recent) symptoms was chosen based on data regarding serum NfL levels in stroke, as this is a monophasic neurologic injury, showing return to levels of healthy controls at around 6 months post-injury (21). An additional 20 patients with a primary headache disorder such as migraine (excluding patients with idiopathic intracranial hypertension) were included as non-inflammatory neurologic controls.

Demographics of each patient and associated autoantibodies, neurologic symptoms, and results of diagnostic testing at the time of initial presentation were obtained through retrospective chart review.

Autoantibody Detection

The presence of serum and CSF autoantibodies to neuronal autoantigens included NMDAR, LGI1, DPPX, GAD65, GlyR, GFAP, TRIM46, and GABA-aR. All antibodies were tested at Mayo Clinic Laboratories with the exception of GABA-aR antibody testing, which was tested at Hospital Clinic, University of Barcelona, Spain. GlyR, GFAP, and TRIM46 antibody testing was performed on cell-based assay (CBA) on a research basis, while all other antibody testing at Mayo clinic was performed on a commercially available basis at that time; all neuronal cell surface antigens (NMDAR, LGI1, DPPX) were performed using CBA.

NfL Analysis

NfL was tested in retrospective and prospective samples, including 26 plasma and 14 CSF samples of patients with AINDs, and 20 control patients. CSF was collected, centrifuged immediately to remove cells and stored at −80°C until analysis. Plasma was obtained in sodium citrate tubes then aliquoted at room temperature and stored at −80°C. Measurement of NfL concentration was performed in duplicates for all samples using the SIMOA Nf-light kit® (Quanterix SR-X™ by Simoa® platform).

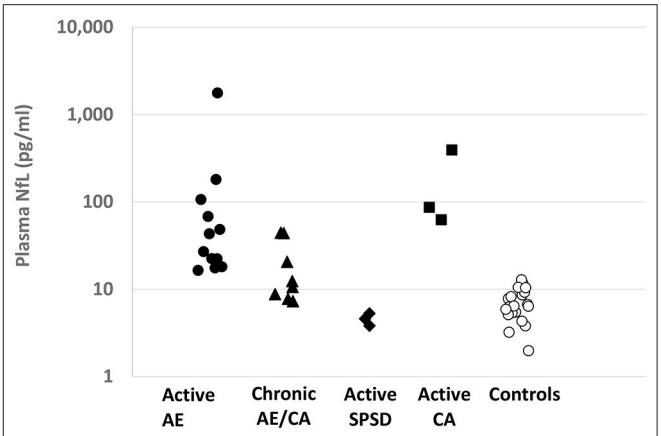

FIGURE 1 | Plasma NfL levels by Autoimmune Neurological Disease Category: Plasma NfL levels (pg/ml) are provided for each defined patient group on a logarithmic scale. AE, autoimmune encephalitis; CA, cerebellar ataxia; SPSD, stiff person spectrum disorder.

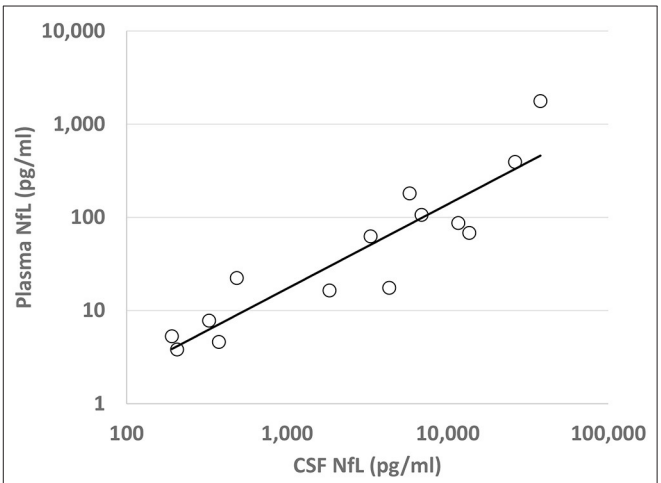

FIGURE 2 | Plasma and Cerebrospinal Fluid NfL Correlation: Correlation of transformed ($\log_{10}$) plasma and CSF NfL levels (pg/ml) for each patient with an AIND, with regression line of best fit shown. $R^2 = 0.83$, $p < 0.0001$.

Theory/Calculation

Plasma and CSF NfL were logarithmically transformed to reduce skew. By group, summary statistics are presented for plasma NfL (**Figure 1**) and differences in mean among groups were analyzed with an ANOVA type model. Different groups were permitted to have different residual variances, and denominator degrees of freedom were determined by the Satterthwaite method. Omnibus F tests tested whether there were any mean differences among all groups and the non-control groups. Pair-wise comparisons were performed with *T*-tests, with the Tukey-Kramer adjustment considered to control the family-wise error rate for all pair-wise

TABLE 1 | Demographic and clinical data of patients.

	Active AE	Chronic AE / CA	Active SPSD	Active CA	Controls
Number of patients	12	8	3	3	20
Age, years, median (range)	64 (23–78)	64 (18–78)	49 (39–55)	75 (63–78)	48 (27–63)
Female, n (%)	6 (50)	6 (75)	1 (33)	3 (100)	14 (70)
Neurologic symptoms at onset of disease, *n* (%)					
Cognitive dysfunction	12 (100)	7 (88)	SPSD: 3 (100)	Ataxia: 3 (100)	Headache: 20 (100)
Psychiatric symptoms	5 (42)	3 (38)			
Seizures	5 (42)	3 (38)			
Ataxia		1 (13)			
Abnormal diagnostic testing*, *n* (% of performed tests)					
MRI	8 (67)	4 (50)	0 (0)	1 (33)	1 (6)
EEG	8 (80)	5 (83)	-	-	-
CSF	10 (91)	3 (43)	1 (33)	3 (100)	3 (16)
Antibody status, *n* (%)					
	NMDAR, 1 (8) LGI1, 2 (17) GAD65, 2 (17) GlyR, 1 (8) GFAP, 1 (8) LE, 2 (17) Ab negative AE, 3 (25)	NMDAR (AE), 1 (13) NMDAR (isolated ataxia), 1 (13) LGI1, 1 (13) DPPX, 1 (13) GABA-aR/GAD65, 1 (13) GAD65, 1 (13) Ab negative AE, 2 (26)	GlyR, 2 (66) LGI1, 1 (33)	GAD65, 1 (33) TRIM46, 1 (33) Paraneoplastic cerebellar degeneration, 1 (33)	N/A
Plasma NfL, pg/ml, geometric mean (range)	51.4 (16.4–1,768)	15.1 (7.3–44.4)	4.5 (3.8–5.3)	128.8 (62.5–393.4)	6.4 (2.0–12.8)
CSF NfL, pg/ml, median (range)	1,161 (486–37,818)	327	207 (192–376)	11,650 (3,311–26,274)	476 (159–3,423)

**MRI abnormalities included unilateral or bitemporal T2 hyperintense signal, parenchymal or leptomeningeal contrast enhancement, hippocampal atrophy, cerebellar degeneration, and extensive white matter disease. EEG abnormalities included diffuse or focal slowing, epileptiform discharges, or electrographic/electroclinical seizures. CSF abnormalities included >5 nucleated cells /mm^3, >2 unique CSF oligoclonal bands, or protein > 50 mg/dl.*

comparisons. For a subset of non-control patients where CSF NfL was available, Pearson correlations were run for plasma and CSF NfL (**Figure 2**). Analysis was performed in SAS 9.4, STATA 15.1, and R 3.6.1.

RESULTS

Patient demographics and clinical presentations are summarized in **Table 1**. Patients with either active AE or CA tended to be older [median 64 years-old (yo) and 75 yo], compared to those with active SPSD (median 49 yo) and controls (median 48 yo). Patients with chronic AE/CA were also older (median 64 yo) compared to the active SPSD and control groups. However, age demographics between active AE and chronic AE/CA were similar. Diagnostic testing (MRI, EEG, and/or CSF) were abnormal in the majority of patients with encephalitic or cerebellar symptoms. Plasma NfL levels in patients with chronic AE/CA were obtained a median of 10 months after the last episode of symptom worsening (range 7–108 months).

Elevated plasma NfL levels were seen in patients with active AE/CA compared to controls [geometric mean (GM) 51.4 vs. 6.4 pg/ml, $p = 0.002$] as shown in **Table 1**, **Figure 1**. Elevated plasma NfL was seen regardless of presence of MRI abnormalities. The group of patients with chronic AE/CA showed a non-significant trend toward lower plasma NfL levels (GM 15.1 pg/ml) when compared to active AE or active CA [GM 51.4 pg/ml ($p = 0.11$) and 128.8 pg/ml ($p = 0.15$), respectively]. Notably, the three patients with plasma NfL levels above our control range were 7, 8, and 9 months out from their last new or worsening of symptoms (ages of each patient were 46, 78, and 73 yo, respectively); the remainder were 9 or more months out. Active CA also showed elevated plasma NfL (GM 128.8 pg/ml). Active SPSD showed lower plasma NfL (GM 4.5 pg/ml) compared to both active AE ($p < 0.001$) and chronic AE/CA ($p = 0.014$).

A correlation between CSF NfL and plasma NfL was noted for all AINDs ($R^2 = 0.83$, $p < 0.001$), as shown in **Figure 2**. For patients with active AE, no significant correlation was found between the initial plasma NfL and the Modified Rankin Score (mRS) at 1 year after presentation ($R^2 = 0.404$, $p = 0.06$, $n = 9$), nor was a correlation found for CSF NfL ($R^2 = 0.420$, $p = 0.16$, $n = 6$).

Six patients had subsequent prospective longitudinal samples obtained. These individual patients and the trend of the plasma

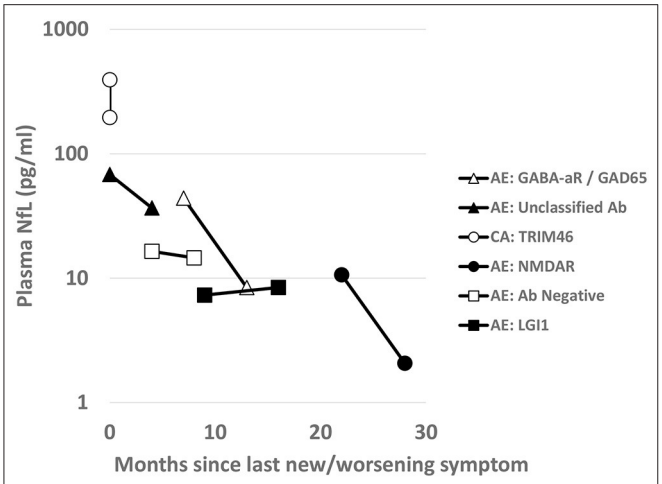

FIGURE 3 | Longitudinal Plasma NfL: For the six AIND patients with longitudinal plasma sample collections, NfL levels (pg/ml) are shown on a logarithmic scale over time. Scale shows collections over months since onset of neurological symptoms or time since last clinical relapse of their disease. Between the first and second time point for each sample patients were treated with variable immunotherapies (TRIM46 with steroids with only 3 weeks in between sampling; unclassified antibody with steroids and rituximab; antibody negative AE with steroids and rituximab; for the remainder [NMDA, LGI, GABA-aR/GAD65], they were on maintained on rituximab therapy.

NfL levels for each AIND relative are shown in **Figure 3**. Each patient had immunotherapy initiation close to initial sample collection (range 3 months prior to 1 month after sample collection).

DISCUSSION

In this study, we used a highly sensitive assay to detect NfL levels in the plasma of patients with AINDs–including active AE, active SPSD, active CA, and chronic AE/CA.

We demonstrated a statistically significant elevation in plasma NfL levels in patients with active AE compared to our controls ($p = 0.002$) and those with SPSD ($p < 0.001$). Similar to other published studies, NfL levels tested at follow up during chronic disease tended to reflect lower NfL levels; however, the majority of these studies focused on CSF NfL (17–19) with one study demonstrating this trend in 13 follow up samples (20).

Looking at the trajectory of plasma NfL for individual patients with AINDs after initial immunotherapy, we can see that each individual demonstrated a different downtrend of plasma NfL after immunotherapy initiation. The differences in trajectory may be due to sampling times (decrease may not be linear), differences in demographics, AIND subtype, or immunotherapy regimen. It is notable that NfL may remain elevated relative to our neurologic control patients for > 1 year after immunotherapy initiation; this has been noted in patients after a monophasic traumatic brain injury as well (22–24). Further studies may look at whether this is a chronic state or if the NfL level would decrease further with additional immunotherapy, and if differences in trajectories for a similar AIND would be seen between different immunotherapy regimens.

We were not able to demonstrate a prognostic value of plasma or CSF NfL in our cohort for active AE–there was no significant correlation between these and the 1-year mRS. This may have occurred for several reasons: (1) a variety of autoantibody and encephalitic syndromes were included in our analysis–it may be that NfL levels vary too widely between these different syndromes, and while it may be prognostic for one syndrome, it may not be prognostic for all. Clinical scores such as the anti-NMDAR encephalitis 1-year functional status (NEOS) score have been developed recently to assist in prediction of 1-year functional status (25)–but even these has only been validated in cohorts of anti-NMDAR encephalitis; (2) It may be that initial NfL levels are prognostic, but our small sample size limited our ability to detect this; or (3) it may be that these levels are not prognostic, with other factors such as duration of symptoms, age, specific autoantibody syndrome, or specific immunotherapy regimens giving greater weight to eventual functional outcome (26).

We did not have any patients with longitudinal sampling have a significant clinical relapse during the study period, so we were not able to assess the predictive values of initial NfL on risk of subsequent relapse. There was not a difference noted between plasma NfL levels in active encephalitic symptoms in initial presentation verses relapse, although longitudinal data prior to relapse in this cohort was lacking. In stable patients on maintenance therapy for AE, having a predictive biomarker for subsequent relapse risk would have great utility. As older age may correlate with both outcome of AE (26) and NfL levels, further longitudinal studies looking at NfL levels, relapse rates, and functional outcomes in a defined age range may be of use in determining the predictive value of NfL.

For the other AINDs investigated in this study, the active autoimmune ataxias showed similar or greater elevations in plasma NfL levels–this is likely due to a greater number of paraneoplastic syndromes within this group. Paraneoplastic cerebellar ataxia syndromes thought to be largely T-cell mediated, may cause greater neuronal destruction. In the cases of SPSD, while the exact pathophysiology remains elusive, it is thought that impairment of the GABA inhibition pathway leading to motor hyperactivity plays a key role in the symptomatology. Therefore, NfL, as an indicator of neuronal injury, may not be a reliable biomarker for SPSD without the presence of encephalitic symptoms.

Our findings support the use of plasma NfL as a potential minimally-invasive biomarker for disease activity in patients with AINDs with CNS involvement. Our study was limited by several factors, most prominent of which are the relatively small sample size, the heterogeneity of the AINDs studied, the confounder of age between groups studied, and the irregular intervals of longitudinal sampling. Given the small sample size, it may be that our analysis was weighted toward particular AE or AIND subtypes or that it was weighted due to greater prevalence of a particular confounder. If this was the case, our sample may not be indicative of the AE or AIND population as a whole, limiting the generalizability of our individual findings.

The breadth of the AINDs studied represents a trade-off between sample size and homogeneity of the AINDs. For our study, we chose a broader inclusion of AINDs, and worked to show characteristics of subtypes of AINDs, analyzing these subtypes individually as able. However, for the analysis of the correlation of CSF and plasma NfL levels and the longitudinal response of plasma NfL to treatment, we included all AINDs to provide appropriate level of detail in analysis. It may be that these relationships hold for only certain AIND subtypes–such as AE or autoimmune cerebellar ataxias, or for NSAS alone, or for individual autoantibody syndromes. Studies including a larger population of each AIND or individual autoantibody syndrome may be able to better define and confirm these relationships as being specific for an AIND subtype or generalizable.

Age and NfL levels have a known correlation–NfL levels increase as age increases. While we were not able to completely age and gender-match our AIND population to healthy controls, we did have a non-inflammatory neurological disease control population in patients diagnosed with primary headache syndromes, in addition to a small chronic AE/CA cohort with similar age demographics to our active AE cohort. One limitation in this study, however, is that the non-inflammatory headache control population was a younger age group (median 48 yo, range 27–63 yo) when comparing to the AE/CA study population. It is notable that all patients with active AE had plasma NfL levels higher than those found in all age groups (range 20–68 yo) in a study measuring serum NfL levels in 79 healthy individuals (27).

Another limitation of this study is the retrospective, cross-sectional design. For our non-active AE/CA patient group, we did not have longitudinal samples for each patient to evaluate the trend of plasma NfL levels over time. For the three patients with plasma NfL levels above our control range, patients were 7, 8, and 9 months out from their last new or worsening of symptoms and the ages of each patient were 46, 78, and 73 years old respectively. For the 46-year-old-female with non-active GABA-aR AE, her NfL level continued to trend downward at 12 months. For the other two patients at 8 and 9 months, it is unclear if they would have continued to have this downward trend in their NfL levels or if these higher levels represent an underlying chronic neurodegeneration, particularly at the ages of 78 and 73 years old. Additionally, chronically high levels of NfL could represent a prognostic biomarker for persistent neurobehavioral symptoms perhaps related to a chronic neurodegenerative process following AE or CA. In a study measuring exosomal and plasma levels of NfL in mild TBI, those injures associated with higher NfL levels, even years after injury, the greatest elevation were seen in those patients with ongoing neurobehavioral symptoms including postconcussive syndrome, posttraumatic stress disorder and depression (28).

Follow-up sampling for each of our patients was conducted during in-person standard of care visit clinic visits when available–this resulted in irregular sampling intervals for the longitudinal analysis of NfL. To best define NfL as a therapeutic biomarker, regular sampling must be performed to understand the trend of NfL under states of recovery, relapse, and progression and compare between various immunotherapy strategies. For these reasons, larger prospective studies, ideally with standardized intervals of sampling, are needed to understand the longitudinal relationship between NfL and the clinical features, disease severity and long-term outcomes of specific AE and other AINDs.

CONCLUSIONS

Our findings support the potential use of plasma NfL as a minimally-invasive diagnostic and therapeutic biomarker for AINDs with CNS involvement. Further larger, prospective studies are warranted to evaluate the use of NfL in AE and AINDs with the potential to influence decision-making regarding the selection and escalation of immunotherapy and to inform the monitoring and recovery of patients with AE and AINDs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved by the Institution Review Board of the University of Colorado, Aurora, CO. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and implementation of the research and to the analysis of the results. SS, RK, CM, and AP provided the statistical analysis. CM contributed to sample collection and preparation. CM and AR carried out biomarker and sample testing. AP and RK wrote the manuscript. All authors contributed to review and revisions of the manuscript.

FUNDING

This study was supported by the Rocky Mountain MS Center, the Drake family in the name of Susan Drake and grant funding from the Department of Neurology at the University of Colorado.

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Conflict of Interest: RK has received compensation for advisory boards and consultancy with Genentech/Roche. EA has received compensation for activities such as advisory boards, lectures and consultancy with the following companies and organizations: Actelion/Janssen, Alexion, Bayer, Biogen, Celgene/BMS, EMD Serono/Merck, Genentech/Roche, Genzyme. EA has received research support from the following: Biogen, Genentech/Roche, Novartis, TG Therapeutics, Patient-Centered Outcomes Research Initiative, National Multiple Sclerosis Society, National Institutes of Health, and Rocky Mountain MS Center. KN has received grant funding from Genentech and Novartis and consulting fees from Novartis, Biogen, and Bristol Meyers Squibb. TV has received compensation for lectures and consultancy from Biogen, Genentech/Roche, Siranax, Celgene, EMD Serono and Novartis and has received research support from Rocky Mountain Multiple Sclerosis Center, Celgene, Biogen, Anokion, Genentech, F. Hoffmann-La Roche Ltd, GW Pharma and TG Therapeutics, Inc. JB reports personal fees from Roche, personal fees from Genentech, personal fees from Viela Bio, personal fees from Chugai Pharma, personal fees from Alexion, grants and personal fees from Novartis, personal fees from Genzyme, personal fees from Clene Nanoscience, personal fees from Mitsubishi-Tanabe, personal fees from Reistone Bio, grants from National Institutes of Health, outside the submitted work. JB has a patent Aquaporumab issued. AP has received research funding from the Drake Family, Rocky Mountain MS Center, and the University of Colorado through the intradepartmental grant. Outside of this work, AP reports honorarium from MedLink and consulting fees from Genentech/Roche and Alexion.

The remaining 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.

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