An Opinion on the Clinical Laboratory Testing following the New 2021 PNS-Care Diagnostic Criteria

Hans Frykman [a,b,*] **and Pankaj Kumar**[b]

The 2004 guidelines on Paraneoplastic Neurological Syndromes (PNS) have recently been updated by a Worldwide PNS-Care expert group (1, 2). The new 2021 PNS-Care guidelines divide these rare and hard to diagnose syndromes into "high risk" and "intermediate-risk" phenotypes as well as the autoantibodies (AAbs) that nearly always accompany them, into "high risk," "intermediate-risk," and "low-risk." The term "high-risk antibodies" replace the old term "onconeural antibody" and the level of risk relates to the risk of a concomitant cancer being present. The presence of the following "high-risk AAbs" are >70% of the time associated with cancer; Hu, CV2, YO, PCA2/MAP1B, SOX1, Amphiphysin, RI/ANNA2, MA2, Tr (DNER), and KLH11 (2). The "intermediate-risk AAbs," where 30%–70% of cases being associated with cancer are; NMDAR, GABAbR, AMPAR, mGLuR5, VGCC, and CASPR2 (with Morvan syndrome), and the "low-risk AAbs" associated with cancer in <30% cases are; LGI1, DPPX, mGluR1, GABAaR, GFAP, GAD65, and CASPR2 (without Morvan syndrome). According to the new guidelines, the antibody status guides further workup depending on the age, sex, and clinical phenotype, which ultimately leads to the diagnosis. For example, the true presence of a high-risk antibody that often predates the cancer in that patient inadvertently sets off an intense chase to find that for the antibody typical cancer. Also, the presence of an appropriate antibody is required for the definite diagnosis of PNS in all cases except opsoclonus-myoclonus. This pressures the laboratory further to find the accurate antibody. In addition, the presence of cancer and typical clinical phenotype are also mandatory for the definite diagnosis of PNS.

Several new developments since 2004 have triggered these updated PNS guidelines. Most importantly, the discovery of several new intraneuronal targets, and of pathogenic surface targets with specific antibodies that can occur without cancer. A very important discovery in recent years was the autoimmune encephalitis (AE) group of diseases, serious, underdiagnosed, immune-mediated, inflammatory conditions of the brain. AE is a heterogeneous group of disorders, associated with AAbs that targets intracellular antigens, synaptic receptors, or cell-surface (ion channels) proteins. AE generally respond well to early immunotherapy. The clinical diagnosis is often difficult, and the

[a]Department of Medicine, University of British Columbia, Vancouver, BC, Canada; [b]BC Neuroimmunology Laboratory, Vancouver, BC, Canada.
*Address correspondence to this author at: Department of Medicine, University of British Columbia, Vancouver, British Columbia V6T 2B5, Canada. E-mail hans.frykman@ubc.ca.
Received June 6, 2021; accepted October 28, 2021.
https://doi.org/10.1093/jalm/jfab154

suspicion is typically raised when the onset of neuropsychiatric symptoms is subacute usually within a few weeks but <3 months and not readily explained by a previous medical condition. Early antibody testing, using a reference laboratory is immensely helpful in making clinical decisions on the diagnosis and treatment. The incidence of AE diagnosis rises drastically in the vicinity of such reference laboratories and likely remains underdiagnosed in other areas (3, 4). Correct early diagnosis with the finding of an antibody is associated with significantly better outcomes and much lower overall health care costs (5). AE can be further classified according to the combination of the following: clinical presentations, brain region involved (e.g., limbic, cerebellar and sensory), cancer association, CSF and AAbs profile, and pathophysiological mechanism (e.g., intracellular and/or cell-surface synaptic antigens) (6). Importantly, the neuropsychiatric symptoms can occur in the presence of pathogenic AAbs with/without cancer. The most frequent and best-characterized forms of AE are anti-NMDA receptor (NMDAR) and LGI1encephalitis (7). Notable breakthroughs in recent years have identified many new AAbs, especially the neuronal-surface Abs that are directly pathogenic, including GABAbR, AMPAR, DPPX, and CASPR2. The list is rapidly expanding.

On the contrary, "high-risk PNS" previously known as classic PNS and accompanying "high-risk AAbs," previously onconeural AAbs; These include Hu/ANNA1, CV2/CRMP5, YO/PCA1, and SOX1 AAbs that target intracellular antigens and are not considered to be directly pathogenic. If correctly measured these AAbs are biomarkers guiding the search for the underlying cancer (2). Importantly, probability of a specific cancer is dependent on age, sex, and ethnic background. This highlights the importance of early detection of cancer AAbs to guide early interventions and improved outcomes for PNS (4). Of special note, with the rise of immune checkpoint inhibitors (ICI), the incidence of PNS has increased substantially. It is now understood that ICI can worsen already present subclinical PNS and can help to trigger PNS de novo (8).

Regarding detection of AE AAbs, the tissue-based assays (snap-frozen rat brain immunohistochemistry/immunofluorescence assays, IHC/IFA) followed by cell-based assays (CBA) with fixed or live cells and/or immunoblots, depending on the antigen, have been the adopted gold standard in the few laboratories considered as reference laboratories, and these techniques are not generally available in other clinical laboratories (2, 9). The rat brain IHC/IFA offers excellent sensitivity and specificity for screening multiple AAbs, however, due to the complex and labor-intensive protocol (7-day protocol; brain-extraction, fixation, cryopreservation, snap-freezing, and cryo-sectioning) to retain the targets in the closest natural configuration and specialized expertise required to identify the reactivity patterns, they are currently available in only a few highly specialized neuroimmunology laboratories (2, 9). More commonly, clinical laboratories use commercial fixed CBA kits for detecting AE AAbs; however, these commercial kits when used alone lack the necessary sensitivity and specificity with >12% positive cases missed and risk for overdiagnosis (9). Commercial kits that test multiple antigens may be useful for STAT testing especially neural-surface AAbs, however, these kits have been shown to linked with false-negative results in a substantial number of patients, mainly those affected by anti-LG1, GABABR or AMPAR encephalitis (2, 10). Most clinical laboratories use the commercial line blots, expressing recombinant proteins, for detecting high-risk AAbs. While these line blots can be a useful screening tool, the number of false positives has shown to be surprisingly high, particularly for well-established "high-risk AAbs" including Yo, CV2, Ma2, ZIC-4, and SOX1 that can lead to overdiagnosis, unwarranted workups, and possibly unnecessary treatments (4, 10–13).

To assure the highest specificity and sensitivity, we recommend a comprehensive orthogonal testing algorithm, in all suspected patients, in which

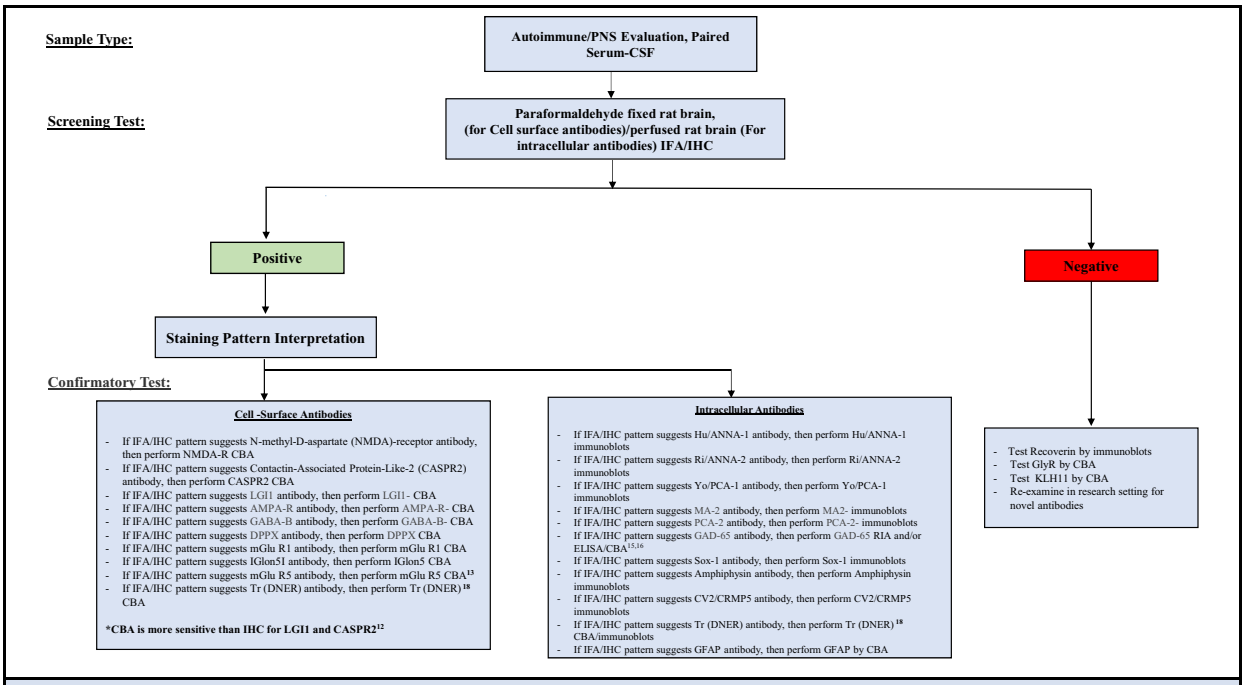

Fig. 1. Our recommendations on comprehensive orthogonal testing algorithm in case of clinically suspected PNS.

panels of AAbs should be analyzed by combining immediate screening using paraformaldehyde fixed snap-frozen rat brain IHC/IFA with CBA for neuronal-surface AAbs and concomitantly analyze with perfused rat brain IHC/IF and immunoblots for "high-risk AAbs." The correct interpretation of tissue staining pattern is extremely important when reflex-testing, to ensure that IHC staining is in fact concordant with the relevant antibody. Figure 1 describes a comprehensive orthogonal testing algorithm that we recommend in our clinical laboratory (Fig. 1) (14–20).

Testing of cerebrospinal fluid (CSF) is mandatory for NMDAR and other neuronal-surface AAbs as it is more sensitive and specific for almost all. The LGI1 AAbs is a proven contradiction, where serum testing is more sensitive and CSF has 53% less sensitivity as a comparison (9), resulting in testing CSF alone may lead to false-negative results for LGI1 AAbs. Therefore, we recommend performing AAbs testing in paired serum-CSF samples concomitantly. In case of an unexpected positive or negative result, e.g., neuronal-surface AAbs positive in serum but negative in CSF, retesting of the sample should always be considered. While snap-frozen rat brain IHC/IF screening can potentially discover novel AAbs, in the case of anti-GlyR, and anti-Recoverin, the rat brain sections have limited utilities, thus any IHC negative samples with classic PNS symptoms should be further tested for the presence of these AAbs by immunoblots or CBA. Similarly, a confirmatory CBA should also be performed in case of IHC positive and immunoblot negative samples with classic PNS symptoms. Figure 2 represents antibody staining patterns for common neuronal antigens such as Hu/ANNA-1, Yo/PCA-1, AMPAR, and NMDA by IFA.

In addition, the CBA is generally considered more sensitive than IHC for LGI1 and CASPR2-AAbs (13). Previous and recent publications have

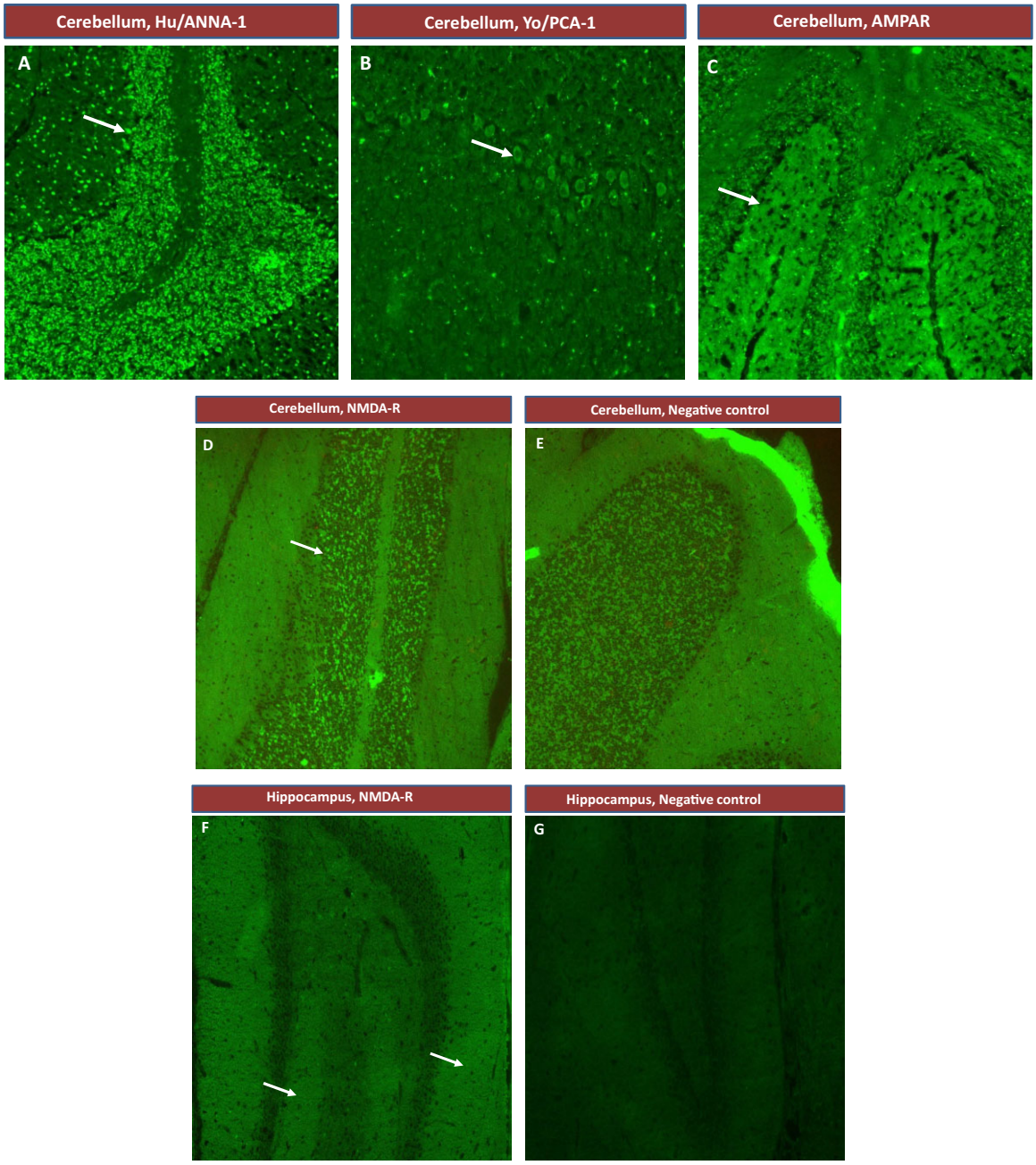

Fig. 2. Represents neuronal autoantibodies testing with IFA technique. IFA of rat brain cerebellum using serum and/or CSF from patients with antibodies to Hu (A), Yo (B), AMPAR (C), *N*-methyl-D-aspartate (anti-NMDA) receptor (D), and a normal individual (E) and IFA of rat brain hippocampus using serum and/or CSF from patients with antibodies to anti-NMDA (F), and a normal individual (G). Note the arrows pointing to areas of IHC staining/reactivity; e.g., Hu antibodies (intracellular staining with intense granular fluorescence in almost all nuclei; (A), Yo (cytoplasmic staining of Purkinje cells; (B), AMPA (intense molecular layer staining; (C), NMDA (intense granular layer staining; and (D) in rat brain cerebellum. In contrast, NMDA Ab reacts intensely with dentate gyrus of hippocampus (the arrows pointing to the areas of reactivity in the molecular layer) of rat brain (F). Lack of staining present with negative controls (E) and (G).

also demonstrated the clinical utility of GAD65 cutoff by RIA and ELISA, although the IHC and/or CBA may be used as corroborative cut-offs particularly in CSF of patients with typical GAD associated phenotype (16, 20).

The choice of testing specific antigens should be based on the observed IHC/IF patterns combined with the very important clinical information such as patient's demographics, neurological syndromes, and cancer association. Any AAbs positive result in CBA and commercial line blots that does not match the IHC/IF patterns or with available clinical picture, are considered questionable and should be investigated further. In almost every case of positive antibody finding the treating neurologist should be contacted to assure a higher likelihood of correct final diagnosis. It is also highly helpful to discuss each case on weekly "clinical rounds" inside the reference laboratory with PNS interested neurologists, clinical pathologists, scientists, and laboratory technicians present.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:* **Employment or Leadership:** H. Frykman, founder and medical director of BC Neuroimmunology Laboratory in UBC Hospital in Vancouver, BC. **Consultant or Advisory Role:** None declared. **Stock Ownership:** None declared. **Honoraria:** None declared. **Research Funding:** None declared. **Expert Testimony:** None declared. **Patents:** None declared.

Acknowledgments: Authors would like to thank Dr. Joel Oger for his insightful suggestions.

References

1. Graus F, Delattre JY, Antoine JC, Dalmau J, Giometto B, Grisold W, et al. Recommended disgnostic criteria for paraneoplastic neurological syndromes. J Neurol Neurosurg Psychiatry 2004;75:1135–40.
2. Graus F, Vogrig A, Muñiz-Castrillo S, Antoine J-CG, Desestret V, Dubey D, et al. Updated diagnostic criteria for paraneoplastic neurologic syndromes. Neurol Neuroimmunol Neuroinflamm 2021;8:e1014.
3. Vogrig A, Gigli GL, Segatti S, Corazza E, Marini A, Bernardini A, et al. Epidemiology of paraneoplastic neurological syndromes; a population-based study. J Neurol 2020;267:26–35.
4. Hébert J, Riche B, Vogrig A, Muñiz-Castrillo S, Joubert B, Picard G, et al. Epidemiology of paraneoplstic neurological syndromes and autoimmune encephalitis in France. Neurol Neuroimmunol Neuroinflamm 2020;7:e883.
5. Cohen J, Sotoca J, Gandhi S, Yeshokumar AK, Lipkin EG, Geocadin RG, et al. Autoimmune encephalitis: a costly condition. Neurology 2019;92:e964–e972.
6. Graus F, Titulaer MJ, Balu R, Benseler S, Bien CG, Cellucci T, et al. A clinical approach to diagnosis of autoimmune encephalitis. Lancet Neurol 2016;15:391–404.
7. Dalmau J, Tüzün E, Wu H, Masjuan J, Rossi JE, Voloschin A, et al. Paraneoplastic anti-*N*-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Ann Neurol 2007;61:25–36.
8. Graus F, Dalmau J. Paraneoplstic neurological syndromes in the era of immune checkpoint inhibitors. Nat Rev Clin Oncol 2019;16:535–48.
9. McCracken L, Zhang J, Greene M, Crivaro A, Gonzalez J, Kamoun M, et al. Improving the antibody-based evaluation of autoimmune encephalitis. Neurol Neuroimmunol Neuroinflamm 2017;4:e404.
10. Ebright MJ, Li SH, Reynolds E, Burke JF, Claytor BR, Grisold A, et al. Unintended consequences of Mayo paraneoplastic evaluations. Neurology 2018;91: e2057–e2066.
11. Ruiz-García R, Muñoz-Sánchez G, Naranjo L, Guasp M, Sabater L, Saiz A, et al. Limitations of a commercial assay as diagnostic test of autoimmune encephalitis. Front Immunol 2021;12:691536.
12. Déchelotte B, Muñiz-Castrillo S, Joubert B, Vogrig A, Picard G, Rogemond V, et al. Diagnostic yield of commercial immunodots to diagnose paraneoplastic

neurologic syndromes. Neurol Neuroimmunol Neuroinflamm 2020;7:e701.
13. Gadoth A, Pittock SJ, Dubey D, McKeon A, Britton JW, Schmeling JE, et al. Expanded phenotypes and outcomes among 256 LGI1/CASPR2-IgG-positive patients. Ann Neurol 2017;82:79–92.
14. Lancaster E, Martinez-Hernandez E, Titulaer MJ, Boulos M, Weaver S, Antoine J-C, et al. Antibodies to metabotropic glutamate receptor 5 in the Ophelia syndrome. Neurology 2011;77:1698–701.
15. Saiz A, Blanco Y, Sabater L, González F, Bataller L, Casamitjana R, et al. Spectrum of neurological syndromes associated with glutamic acid decarboxylase antibodies: diagnostic clues for this association. Brain 2008;131:2553–63.
16. Muñoz-Lopetegi A, de Bruijn MAAM, Boukhrissi S, Bastiaansen AEM, Nagtzaam MMP, Hulsenboom ESP, et al. Neurologic syndromes related to anti-GAD65: clinical and serologic response to treatment. Neurol Neuroimmunol Neuroinflamm 2020;7:e696.
17. Kunchok A, Zekeridou A, McKeon A. Autoimmune glial fibrillary acidic protein astrocytopathy. Curr Opin Neurol 2019;32:452–8.
18. Mandel-Brehm C, Dubey D, Kryzer TJ, O'Donovan BD, Tran B, Vazquez SE, et al. Kelch-like protein 11 antibodies in seminoma-associated paraneoplastic encephalitis. N Engl J Med 2019;381:47–54.
19. Dalmau J, Geis C, Graus F. Autoantibodies to synaptic receptors and neuronal cell surface proteins in autoimmune diseases of the central nervous system. Physiol Rev 2017;97:839–87.
20. Dalmau J, Dalakas MC, Kolson DL, Paul F, Zamvil SS. N2 year in review. Neurol Neuroimmunol Neuroinflamm 2021;8:e925.