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Assessing Commercial Tissue-Based Assays for Autoimmune Neurologic Disorders (I)

Antibodies to Intracellular Antigens

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Abstract

Background and Objectives

Current strategies to detect autoantibodies against intracellular neural antigens (IC-Abs) include tissue-based assays (TBAs) alongside line blots or cell-based assays (CBAs). Many clinical laboratories use commercially available TBAs as a screening test, but their diagnostic yield has not been assessed. We determined the performance of 2 commercial TBAs in detecting IC-Abs.

Methods

We analyzed samples from 100 patients with autoimmune or paraneoplastic neurologic syndromes harboring IC-Abs (confirmed by in-house TBAs and line blots or CBAs) and 50 negative controls. IC-Abs samples included serum (10 each for Hu, Yo, Ri, SOX1, CV2, Ma2, Tr, amphiphysin, and GAD65 antibodies) or CSF (10 with GFAP antibodies) samples. Two commercial indirect immunofluorescence (IIF) TBAs (INOVA and EUROIMMUN) were assessed by 2 experienced investigators and 3 less experienced raters, all blinded to antibody status. Discordant results were re-evaluated through interrater discussion and assessed using Cohen's kappa.

Results

The 2 experienced raters showed substantial agreement (85% for INOVA, 83% for EUROIMMUN) on negative/positive results, which increased to >95% after interrater discussion (Cohen's kappa 0.95 and 0.93, respectively). With IIF-INOVA, they correctly identified 118 of 150 samples (79%) and misclassified 28 of 150 (19%, 2 false positives and 26 false negatives) while results remained discordant in the remaining 4 of 150 samples (2%). With IIF-EUROIMMUN, they correctly identified 105 of 150 samples (70%) and misclassified 40 of 150 (27%, 6 false positives, 34 false negatives), with discordance in 5 of 150 samples (3%). Overall, the sensitivity was 73% for IIF-INOVA and 66% for IIF-EUROIMMUN. The specificity was 96% for IIF-INOVA and 88% for IIF-EUROIMMUN. Both TBAs showed low sensitivity in detecting CV2, SOX1, and amphiphysin antibodies while Ma2 antibodies were missed mainly by IIF-EUROIMMUN and Hu/Ri antibodies by IIF-INOVA. Antibody-specific immunostaining patterns were correctly identified in 62 of 100 positive samples with IIF-INOVA and 55

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Glossary

CBA = cell-based assay; **GL** = granular layer; **IC-Abs** = neuronal antibodies against intracellular targets; **IHC** = immunohistochemistry; **IIF** = indirect immunofluorescence; **ML** = molecular layer; **NC** = negative control; **PCs** = Purkinje cells; **TBA** = tissue-based assay; **WM** = white matter.

of 100 with IIF-EUROIMMUN ($p = 0.34$). Less experienced raters showed higher rates of false-positive results (up to 22% with IIF-EUROIMMUN).

Discussion

The performance of commercial IIF-TBAs for IC-Abs detection is suboptimal, exhibiting high false-negative rates of 25%–35%. Therefore, commercial TBAs should not be used alone for IC-Abs screening, but alongside more sensitive techniques, such as line blots. Discordant results between 2 techniques should prompt retesting in reference centers with in-house TBAs, particularly when the suspicion of an autoimmune or paraneoplastic syndrome is high.

Introduction

Autoimmune neurologic syndromes associated with autoantibodies against intracellular (IC-Abs) or neuronal/glial cell surface (NSAbs) antigens are immune-mediated disorders often considered in the differential diagnosis of a wide range of paraneoplastic and neuropsychiatric conditions. IC-Abs are frequently linked to a paraneoplastic etiology, although they can occur without tumor association,[1] while NSAbs are commonly associated with nonparaneoplastic autoimmune encephalitis, despite their frequent association with tumors.[1] Detecting specific antibodies from either category (IC-Abs or NSAbs) is crucial for confirming the diagnosis of these disorders, guiding tumor searches and treatment interventions, and determining long-term prognosis.[1]

For both categories, studies indicate that consistent diagnoses are achieved when antibodies are demonstrated using 2 techniques.[2,3] One technique involves demonstrating neural reactivity in murine or primate brain slices (tissue-based assays [TBAs]) while the other technique, which varies depending on the antibody category, confirms the specific target antigen (specific test). For most IC-Abs, specific tests include line blots or cell-based assays (CBAs), and less frequently, radioimmunoassay or ELISA (e.g., GAD65).[1,4-6] For NSAbs, specific tests are primarily based on CBAs.[7,8]

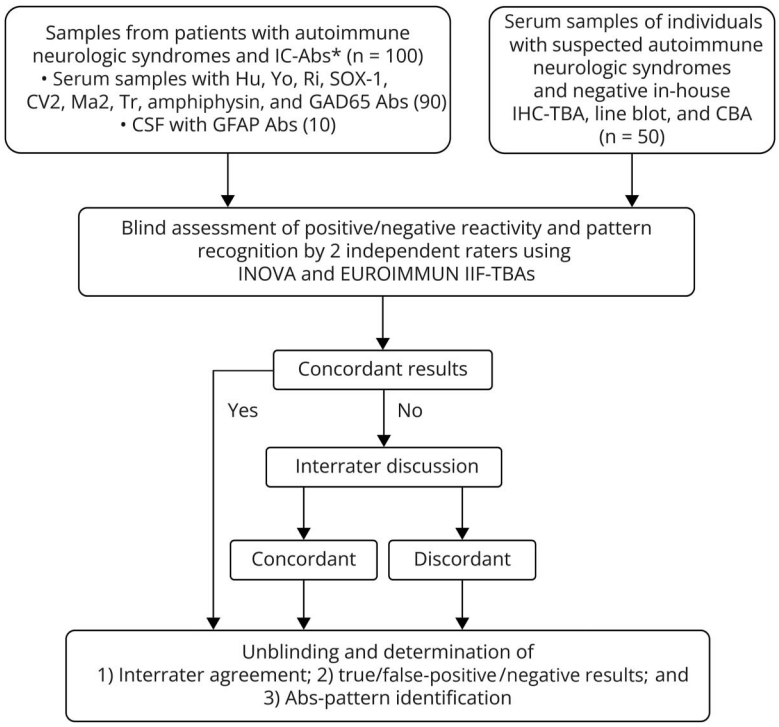
This dual approach, combining TBA with a confirmatory test, is recommended to reduce the risk of false positives or negatives.[3-5,9,10] In referral centers with in-house TBAs, this technique not only confirms the presence of neural-specific antibodies but also provides robust evidence for multiple antibodies or antibodies against novel antigens not detectable with the specific techniques (CBAs or line blots). It also helps identify potential diagnostic errors in specific tests when the findings do not align with the patient's syndrome. Although the limitations of commercial specific tests (such as line blots and CBAs) have been previously reported, the diagnostic value of commercially available indirect immunofluorescence (IIF)-TBAs, which most clinical laboratories depend on, remains unclear. The aim of this study was to evaluate the effectiveness of 2 widely used commercial IIF-TBAs in detecting IC-Abs. In addition, a companion article will assess the performance of a commercial IIF-TBA in identifying NSAbs.

Methods

Patients, Inclusion Criteria, and Sample Selection

Patients were identified from the Neuroimmunology Collection (SCII number C0000051) at IDIBAPS-Hospital Clínic of Barcelona, Spain, which contains clinical data and samples from over 20,000 patients with suspected autoimmune or paraneoplastic neurologic disorders who have been referred to our laboratory for antibody studies between 2007 and 2024. From this collection, we examined 100 samples from patients with autoimmune/paraneoplastic neurologic syndromes and IC-Abs and 50 samples from individuals without autoantibodies (controls) (Figure 1). Patients' samples included 90 serum samples (10 for each of the following antibodies: Hu, Ri, Yo, Tr, GAD65, amphiphysin, SOX1, CV2, and Ma2) and 10 CSF samples with GFAP antibodies. Patients' samples were included if they fulfilled the following criteria: (1) presence of IC-Abs confirmed by 2 techniques: in-house immunohistochemistry (IHC)-TBA showing a distinctive antigen pattern of reactivity with perfused rat cerebellum[11] and line blot (EUROIMMUN – ref. DL 1111-1601-7 G, DL 1111-5001-7 G, DL 1111-6401-7 G, Ravo Diagnostika #PNS14-003) confirming the specific reactivity with the corresponding antigen (Hu, Ri, Yo, Tr, GAD65, amphiphysin, SOX1, CV2, or Ma2), or in-house live CBA for GFAP, and (2) neurologic syndrome consistent with the identified antibody. Only samples for which the experienced raters agreed on a positive staining and recognizable pattern on the in-house IHC-TBA and positivity by line blots/CBAs were selected, whereas samples with multiple IC-Abs and samples

Figure 1 Algorithm of the Study



Abs = antibodies; CBA = cell-based assay; IC-Abs = neural antibodies targeting intracellular targets; IHC-TBA = immunohistochemistry tissue-based assays; IIF-TBAs = indirect immunofluorescence tissue-based assays. *Confirmed by in-house IHC-TBA and a confirmatory test (line blot and/or CBA).

with systemic non-neuronal specific nuclear antibodies (ANAs) were excluded. Control samples were identified from the same Neuroimmunology Collection, showing absence of reactivity with in-house IHC-TBA and negative CBA and line blot.

Detection of IC-Abs by 2 Commercial IIF-TBAs

All samples were tested for reactivity with fixed tissue using 2 commercial IIF-TBAs (EUROIMMUN – ref. FC 1111-1010-8 and INOVA Diagnostics NOVA Lite - ref. 504225). EUROIMMUN IIF-TBA consists of 4 biochips per field containing monkey-derived sections of the cerebellum (gray and white matter), peripheral myelinated nerve, intestinal tissue, and pancreas. INOVA IIF-TBA consists of 3 biochips per field containing sections of monkey cerebellum (white and gray matter), monkey cerebral cortex, and mouse stomach. Tests were performed according to manufacturers' instructions. In brief, diluted serum (1:10 for EUROIMMUN IIF-TBA and 1:80 for INOVA IIF-TBA) and CSF (1:2 for both IIF-TBAs) samples were incubated at room temperature for 30 minutes with the tissue, and reactivity was detected using a fluorescent anti-human antibody (INOVA Diagnostic FITC IgG Conjugate - ref. 508113 for INOVA; included in the kit for EUROIMMUN).

Commercial IIF-TBA results were assessed using a fluorescence microscope (Zeiss Axio Imager M2, Zeiss, Jena, Germany) by 2 expert independent raters (J.D. and F.G.) and 3 less experienced raters (C.M., P.B., and M.S), all blinded to the clinical information and results of the in-house IHC-TBAs. Reactivity was scored as positive or negative by each rater, and in case of positive result, the specific antibody pattern was reported. If no pattern was identified, samples were considered "positive without recognizable pattern." Discrepancies across results were discussed by the 2 expert raters during an interrater discussion. If an agreement was reached, results were considered "concordant." If no agreement was reached, results were considered "discordant." An algorithm of the assessments by the 2 experienced raters is shown in Figure 1. For less experienced raters, results were considered concordant if 2 of 3 raters agreed, without interrater discussion.

Statistical Analysis

Continuous variables (i.e., age) were presented as median with range and categorical variables as proportions and percentages. Interrater agreement on the evaluation of positive/negative results was assessed using the Cohen's kappa for the 2 expert raters and the Fleiss kappa for the 3 less experienced raters. Strength of agreement was considered according to the kappa index value, as reported[12] (≤0.40 was considered poor concordance, 0.41–0.57 fair, 0.58–0.75 good, and >0.75 excellent). For both INOVA and EUROIMMUN IIF-TBAs, the percentages of true/false-positive results and true/false-negative results were determined; sensitivity and specificity were calculated after excluding samples with discordant results. Antibody patterns were analyzed only on samples considered

to have positive reactivity. Diagnostic performance of the INOVA and EUROIMMUN IIF-TBAs was evaluated, after excluding samples with discordant results, based on the following metrics: (1) sensitivity and specificity; (2) McNemar test to evaluate the overall performance regarding positive/negative results; (3) Fisher and chi-square tests to compare the proportion of correct/incorrect patterns. Statistical analyses were performed using R studio (v12.1), PRISM (v9), and medcalc.org.

Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Institutional Review Board of the Hospital Clínic, Barcelona (Reg. HCB/2023/1183). All patients or their proxies provided written informed consent for the storage and use of serum, CSF, and clinical information for research purposes.

Data Availability

Anonymized participant data can be shared with qualified investigators on request to the corresponding author.

Results

Concordance Among Experienced Raters and Performance of Commercial IIF-TBAs

Demographics, main syndromes, and clinical/tumor associations of the 100 patients with IC-Abs were consistent with the expected features for each antibody (eTable 1).

Using the INOVA IIF-TBA, the 2 experienced raters agreed on the negative/positive result in 128 of 150 samples (85%), indicating good agreement (Cohen's kappa = 0.71, 95% CI 0.60–0.81, $p < 0.001$). After reassessing results in the interrater meeting, consensus was reached in 146 of 150 samples (97%) (Cohen's kappa after discussion: 0.95, 95% CI 0.89–0.99, $p < 0.001$), with 4 samples remaining discordant (one SOX1-positive and 2 CV2-positive samples and one negative control).

Using the EUROIMMUN IIF-TBA, the 2 experienced raters were concordant in 124 of 150 samples (83%), showing good agreement (Cohen's kappa = 0.66, 95% CI 0.53-0.77, $p < 0.001$). After reassessing results in the interrater meeting, consensus was reached in 145 of 150 samples (96%) (Cohen's kappa = 0.93, 95% CI 0.87–0.99, $p < 0.001$), with 5 samples remaining discordant (one GAD65-positive, one amphiphysin-positive, and 2 Ma2-positive samples and one negative control).

The INOVA IIF-TBA correctly identified 118 of 150 samples (79%) (true positives/negatives), misclassified 28 of 150 (19%, false positives/negatives), and could not classify 4 of 150 (2%) because of discordant results among raters. When considering only the IC-Abs–positive samples (n = 100), the INOVA IIF-TBA correctly identified 71 of 100 (71%, true positives), missed 26 of 100 (26%, false negatives), and could not classify 3 of 100 (3%) because of discordant results among raters (Figure 2A).

Of note, like the correctly classified samples, the misclassified samples also showed clinical features typically expected based on the underlying antibody (eTable 1).

Among the control samples (n = 50), this test correctly identified 47 of 50 (94%, true negatives), misclassified 2 of 50 (4%, false positives), and could not classify 1 of 50 (2%) because of discordant results among raters (Figure 2A). Overall, the INOVA IIF-TBA showed a sensitivity of 73% (95% CI 63–82) and a specificity of 96% (95% CI 86–99).

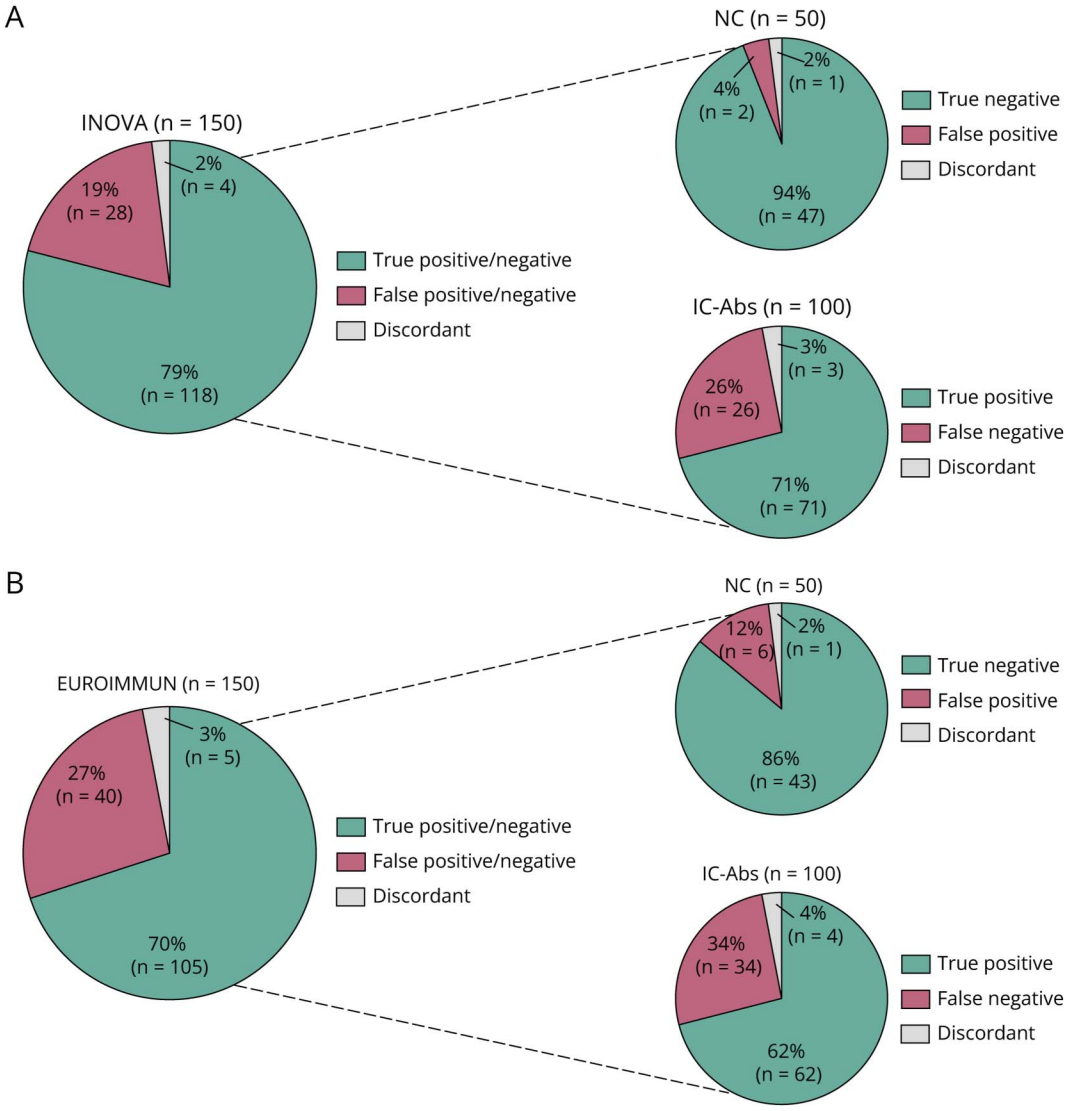
The EUROIMMUN IIF-TBA correctly identified 105 of 150 samples (70%) (true positives/negatives), misclassified 40 of 150 (27%, false positives/negatives), and could not classify 5 of 150 (3%) because of discordant results among raters. Among the IC-Abs–positive samples (n = 100), 62 of 100 (62%) were correctly identified (true positives), 34 of 100 (34%) were missed (false negatives), and 4 of 100 (4%) could not be classified because of discordant results (Figure 2B). Among the control samples, 43 of 50 (86%) were correctly identified (true negatives), 6 of 50 (12%) were misclassified (false positives), and 1 of 50 (2%) could not be classified (Figure 2B). Overall, the EUROIMMUN IIF-TBA showed a sensitivity of 64% (95% CI 54–74) and a specificity of 88% (95% CI 75-54).

No significant differences in overall performance were found between the 2 IIF-TBAs (McNemar test: for negative controls, p value = 0.29; for IC-Abs–positive samples, p value = 0.56).

Identification of Antibody-Specific Immunostaining Patterns

Antibody-specific immunostaining patterns were correctly identified in 62 of 100 positive samples (62%) with INOVA IIF-TBA and 55 of 100 positive samples (55%) with EUROIMMUN IIF-TBA (Figure 3), without significant differences between the 2 IIF-TBAs ($p = 0.34$). Figures 4 and 5 illustrate the expected pattern of reactivity for each antibody and compare the proportions of correctly identified patterns with the 2 IIF-TBAs. Apart from these correctly identified patterns, the experienced raters identified as positive, but attributed the reactivity to a wrong antibody ("positive for wrong antibody"), 7 additional samples with INOVA and 7 with EUROIMMUN (Figure 3, A and B). For example, one sample with Ma2 antibodies showed a diffuse nuclear reactivity similar to that of Hu/Ri antibodies when tested with INOVA IIF-TBA but did not show the expected nucleolar staining of Purkinje cells typically observed in Ma2 antibody reactivity. In this case, although the sample was correctly identified as positive by both raters, the identified pattern was not correct.

Figure 2 Proportion of True and False Positives/Negatives and of Discordant Results With INOVA (A) and EUROIMMUN (B) IIF-TBAs



IC-Abs = neuronal antibodies against intracellular targets; NC = negative controls.

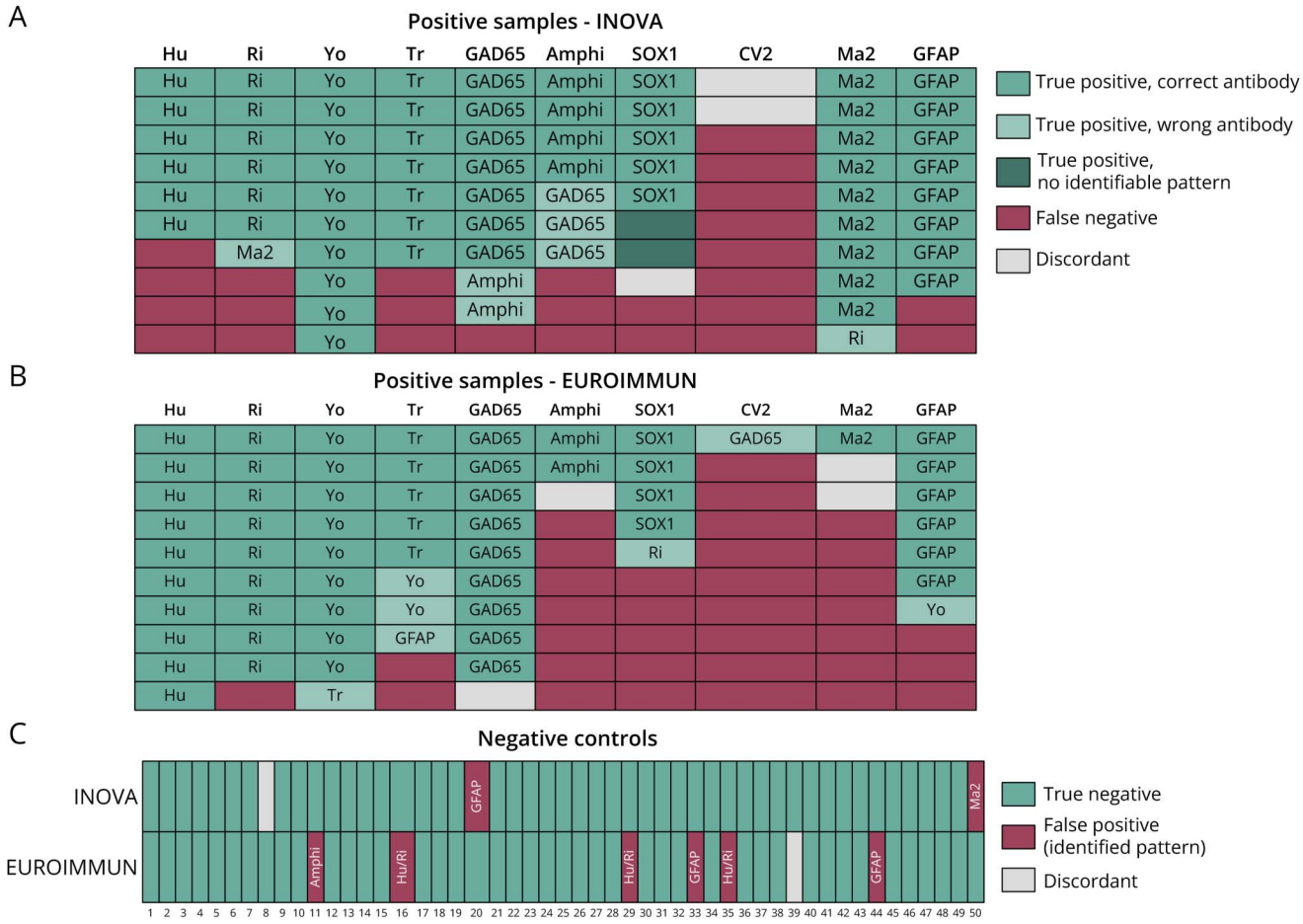
Some important differences were observed concerning specific antibodies (Figures 4 and 5). First, Ma2 antibodies were better detected by INOVA IIF-TBA (which correctly identified 9/10 samples vs 1/10 by EUROIMMUN, $p = 0.001$) while Hu/Ri antibodies were better detected by EUROIMMUN IIF-TBA (which correctly identified 19/20 samples vs 12/20 by INOVA, $p = 0.02$) (Figure 3, A and B, and 4). By contrast, both IIF-TBAs failed to identify CV2 antibodies. Only 1 of 10 CV2 antibody–positive samples was recognized as positive (by the EUROIMMUN kit), but the pattern was attributed to GAD65 antibodies (Figure 3, A and B).

The presence of additional tissues, other than the cerebellum, in the biochips from both IIF-TBAs was helpful in identifying, for example, Ma2 antibodies (which showed a bright nucleolar staining of cortical neurons in the INOVA kit) or GAD65 antibodies (which showed strong reactivity with the pancreatic islets in the EUROIMMUN kit) (Figure 6).

Nonspecific Immunostaining Patterns in Control Samples

Among the 50 control samples, 5 main nonspecific staining patterns were identified in both IIF-TBAs, which may lead to diagnostic errors (Figure 7): (1) neurofilament-like staining pattern (4/50 samples [8%] with INOVA IIF-TBA, 11/50 samples [22%] with EUROIMMUN IIF-TBA), characterized by diffuse axonal staining in the white matter and in the granular layer (GL) of the cerebellum, resembling GFAP antibody reactivity but without the typical radial staining in the molecular layer (ML); (2) blood vessel staining (9/50 samples [18%] with INOVA IIF-TBA, 2/50 samples [4%] with EUROIMMUN IIF-TBA); (3) SOX1-like staining

Figure 3 Heatmap Illustrating the Identified Antibody-Specific Immunostaining Patterns for Each Sample Tested With the 2 Commercial IIF-TBAs



INOVA (A) and EUROIMMUN (B) positive samples are divided into different columns according to the specific antibody. For each antibody column, each of the 10 samples is color-coded according to the results after interrater agreement between the 2 expert raters. In case of samples considered positive, the identified antibody-specific pattern is specified (green: correctly identified as positive for the true antibody; light green: correctly identified as positive but for the wrong antibody; dark green: correctly identified as positive but without an identifiable pattern; wine color: wrongly identified as negative [= false negative]; gray: discordant). (C) Negative controls tested with INOVA IIF-TBA (top row) and EUROIMMUN IIF-TBA (lower row) and the obtained results (green: correctly identified as negative; wine color: wrongly identified as positive; gray: discordant) and identified pattern of reactivity when the sample was considered positive.

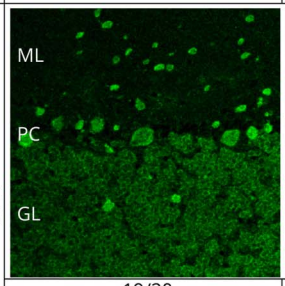
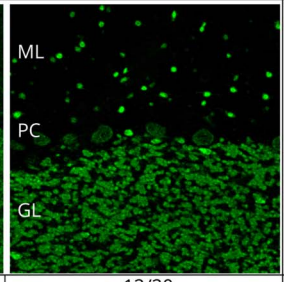
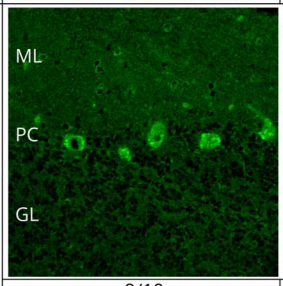
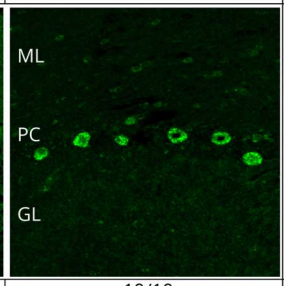
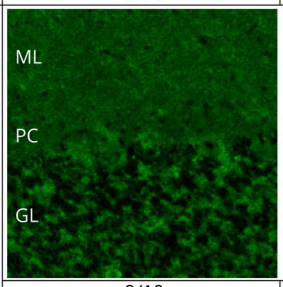
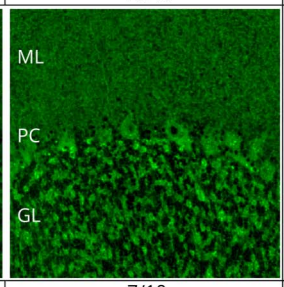
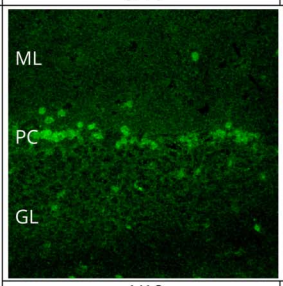
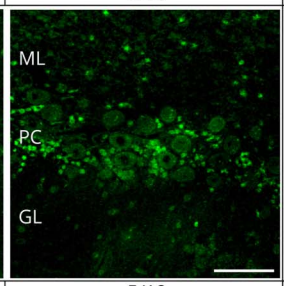
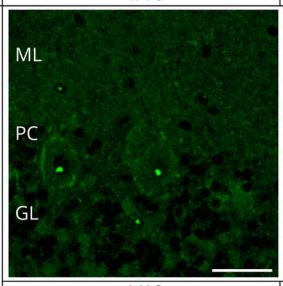
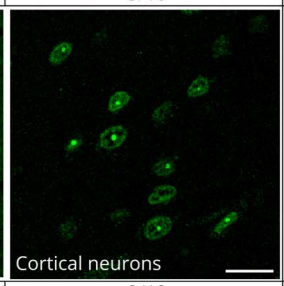
pattern (2/50 samples [4%] with INOVA IIF-TBA, 1/50 samples [2%] with EUROIMMUN IIF-TBA) resembling the reactivity with the Bergmann glia of these antibodies, but without the staining of other isolated glial cells in the white matter; (4) amphiphysin-like staining characterized by a neuropil staining of the ML but with additional diffuse reactivity of the GL and without the typical synaptic bouton pattern of amphiphysin antibodies (1/50 samples [2%] with INOVA IIF-TBA, 13/50 samples [26%] samples with EUROIMMUN IIF-TBA); and (5) Hu/Ri-like staining, characterized by nuclear and cytoplasmic staining of neurons in the GL and ML of the cerebellum (0/50 samples with INOVA IIF-TBA, 3/50 samples [6%] with EUROIMMUN IIF-TBA), with or without concomitant staining of intestinal/stomach tissue.

Although, in most cases, the 2 experienced raters were able to correctly classify these patterns as "nonspecific," a total of 8 negative samples were misclassified as positive: 3 with neurofilament-like staining (2 by EUROIMMUN and one by INOVA), 3 with Hu/Ri-like staining (all by EUROIMMUN), one with amphiphysin-like staining (by EUROIMMUN), and one that was considered to harbor Ma2 antibodies (Figure 3C).

Performance of the 2 Commercial IIF-TBAs by Less Experienced Raters

The 3 less experienced raters agreed on the result (negative/positive) in 121 of 150 samples (81%) (Fleiss kappa: 0.74, 95% CI 0.65–0.82, $p < 0.001$) using INOVA IIF-TBA and in 121 of 150 samples (81%) (Fleiss kappa = 0.74, 95% CI 0.65–0.82, $p < 0.001$) using EUROIMMUN IIF-TBA.

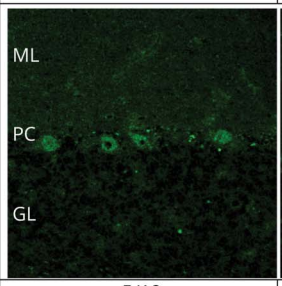
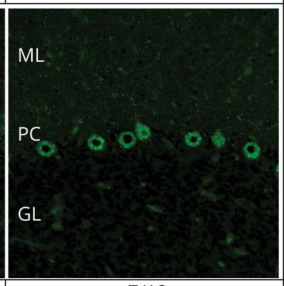
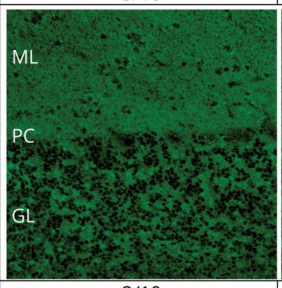
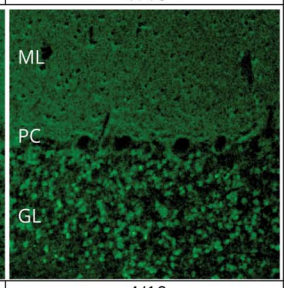
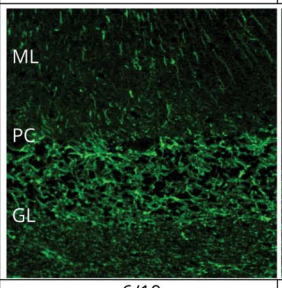
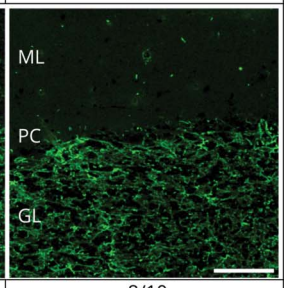
Figure 4 Expected Cerebellar and Additional Tissue Staining in Commercial IIF-TBAs and Comparison Between INOVA and EUROIMMUN for Hu/Ri, Yo, GAD65, SOX1, and Ma2 Antibodies

Expected cerebellar pattern (description)	Correctly identified patterns with EUROIMMUN (n)	Correctly identified patterns with INOVA (n)	*p* Value
Hu/Ri **ML:** Nuclei staining **PC:** Cytoplasm and nuclei staining **GL:** Cytoplasm and nuclei staining			
	19/20	12/20	**0.02***
Yo **ML:** Basket and stellate cells staining **PC:** Cytoplasm staining			
	9/10	10/10	0.99
GAD65 **ML:** Diffuse staining **PC:** Dot-like staining at the base **GL:** Strong diffuse staining, similar to "leopard skin", resembling that of amphiphysin			
	9/10	7/10	0.58
SOX1 Staining of Bergmann glia cells' nuclei within the PC layer			
	4/10	5/10	0.99
Ma2 Staining of nucleoli of cerebellar neurons			
	1/10	9/10	**0.001***

GL = granular layer; ML = molecular layer; PC = Purkinje cell. Scale bar = 100 μm for the images in the first 4 rows; scale bar = 20 μm for the images in the last row.

Among all 150 samples, 121 (81%) were correctly identified as true positives/negatives by at least 2 of the 3 less experienced raters when samples were tested with INOVA IIF-TBA and 102 (68%) when tested with EUROIMMUN IIF-TBA. However, 29 samples (19%) tested with INOVA IIF-TBA and 48 samples (32%)

Figure 5 Expected Cerebellar and Additional Tissue Staining in Commercial IIF-TBAs and Comparison Between INOVA and EUROIMMUN for Tr, Amphiphysin, CV2, and GFAP Antibodies

Expected cerebellar pattern (description)	Correctly identified patterns with EUROIMMUN (n)	Correctly identified patterns with INOVA (n)	*p* Value
Tr **PC:** Cytoplasm and dendrites intense punctate staining			
	5/10	7/10	0.65
Amphiphysin **ML:** Intense diffuse staining **GL:** Diffuse staining, resembling that of GAD65			
	2/10	4/10	0.68
GFAP **ML:** Strong staining of the astrocytes in the white matter, radial pattern in the molecular layer			
	6/10	8/10	0.63
CV2 **ML and GL:** "sand-like fluorescence"	0/10	0/10	0.99

GL = granular layer; ML = molecular layer; PC = Purkinje cell. Scale bar = 100 μm.

tested with EUROIMMUN IIF-TBA were misclassified as false positives/negatives.

Positive samples were correctly identified in 73 of 100 cases (73%) using INOVA IIF-TBA and 63 of 100 cases (63%) using EUROIMMUN IIF-TBA. Antibody-specific staining patterns were correctly identified by at least 2 of the 3 less experienced raters in 58 of 100 samples (58%) using INOVA IIF-TBA and in 54 of 100 (54%) using EUROIMMUN IIF-TBA. In addition to these correctly identified patterns, the less experienced raters identified as positive, but attributed to the wrong antibody ("positive for wrong antibody"), 7 additional samples with INOVA and 5 with EUROIMMUN.

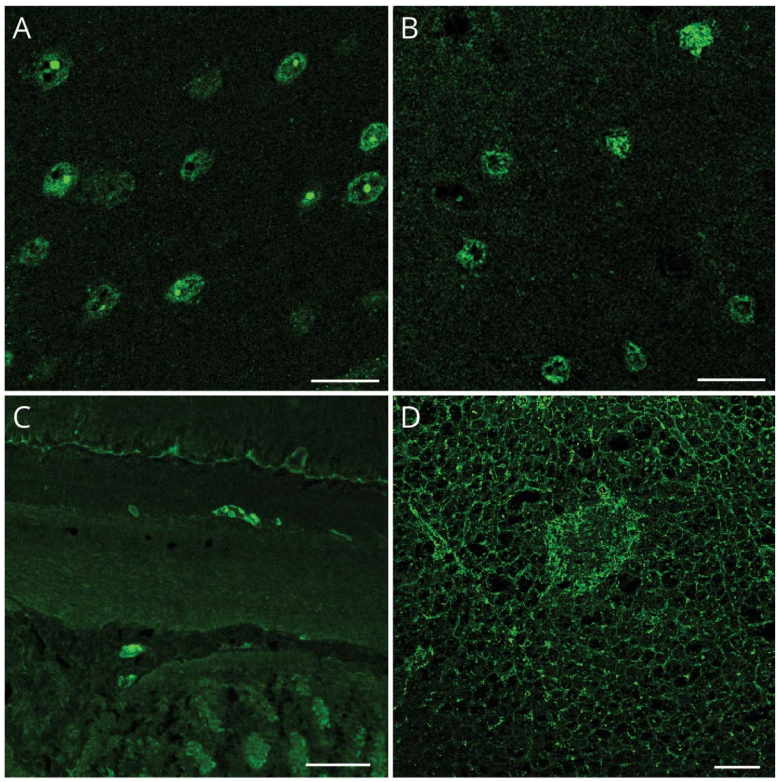
Negative samples were correctly identified in 48 of 50 cases (96%) using INOVA IIF-TBA and 39 of 50 cases (78%) using EUROIMMUN IIF-TBA, whereas they were misclassified (i.e., false positive) in 2 of 50 cases (4%) using INOVA IIF-TBA (1 considered positive for GFAP and 1 for Hu) and 11 of 50 cases (22%) using EUROIMMUN IIF-TBA (5 considered positive for amphiphysin, 3 for GFAP, and 3 for Ri).

The overall sensitivity and specificity of the INOVA IIF-TBA were 73% (95% CI 63–81) and 96% (95% CI 86–100), whereas for the EUROIMMUN IIF-TBA, they were 63% (95% CI 53–73) and 78% (95% CI 64–88), respectively.

Discussion

This study shows that the diagnostic performance of the 2 commercial IIF-TBAs (INOVA and EUROIMMUN) used for testing IC-Abs in patients with suspected autoimmune or paraneoplastic neurologic syndromes is suboptimal, particularly concerning a high rate of false-negative results (low sensitivity). Both IIF-TBAs showed limitations in sensitivity

Figure 6 Main Advantages of Additional Tissues in Both INOVA and EUROIMMUN IIF-TBAs



The presence of cerebral cortex in INOVA IIF-TBA allowed the recognition of Ma2-positive (A) and SOX1-positive (B) samples when the expected cerebellar staining was not clearly visible. The presence of intestinal tissue in EUROIMMUN IIF-TBA (C), similar to the presence of stomach tissue in INOVA IIF-TBA (not shown), allowed demonstration of reactivity with the myenteric plexus in Hu-positive samples. The presence of pancreatic tissue in EUROIMMUN IIF-TBA (D) allowed identification of GAD65 antibodies. Scale bar = 20 μm for (A) and (B); scale bar = 100 μm for (C) and (D).

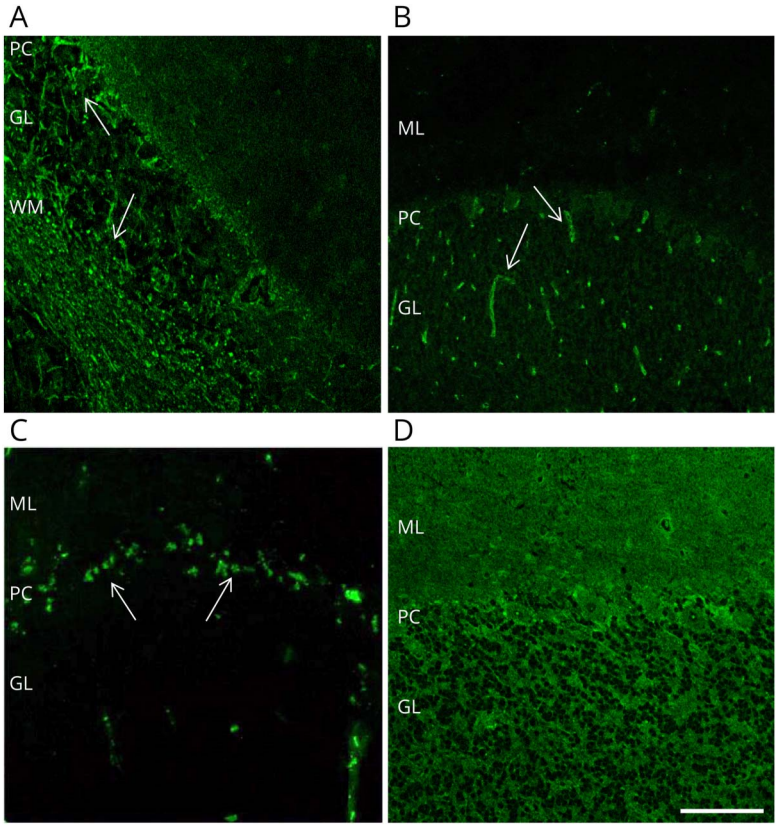
and specificity across a wide range of antibodies, suggesting that they should not be used alone as diagnostic tests. These findings have important clinical implications given that, although the frequency of autoimmune or paraneoplastic neurologic syndromes is low, their inclusion in the differential diagnosis of many disorders is frequent and their definite diagnosis usually depends on the demonstration of neural autoantibodies.

The suboptimal performance of these commercial IIF-TBAs is founded on 3 detected problems: (1) the high rate of false-negative results, (2) the misclassification of some specific antibodies, and (3) the identification of staining patterns in controls that might resemble antibody-specific staining.

Concerning the first problem, these 2 kits allowed detection of positive samples with the correct antibody pattern in only half of the cases (average 5.5/10 for EUROIMMUN and 6.2/10 for INOVA, Figure 3, A and B). Only Yo antibodies with INOVA IIF-TBA and Hu antibodies with EUROIMMUN IIF-TBA were correctly identified in 10 of 10 positive samples. Overall, false-negative results concerned a variety of different antibody targets, including not only the low/moderate-frequency antibodies, such as amphiphysin, SOX1, and CV2, but also more frequent antibodies, such as Hu antibodies (40% missed by the INOVA IIF-TBAs). Possible reasons for not detecting these IC-Abs by both IIF-TBAs, compared with in-house TBAs, include differences in brain tissue fixation, absence of deep cerebellar nuclei, and variable representation of different cerebellar structures/areas (e.g., white matter) across biochips. For instance, previous studies have demonstrated that detection of CV2 antibodies is dependent on the type and depth of brain tissue fixation, as they are detectable when the tissue is deeply fixed (i.e., rat brain intracardially perfused with 4% paraformaldehyde [PFA], as in our in-house TBAs), whereas they are not detected when the tissue is fixed with less stringent procedures (e.g., brain fixation by immersion in PFA or postfixed tissue) or different fixatives (e.g., acetone).[4,13] Although details on tissue processing are not provided by these commercial IIF-TBAs, it is likely that differences in fixation procedures are responsible for the incomplete exposure of the CV2 and GAD65 antigens, contributing to false-negative results. Another concern is that only small portions of the cerebellum (for example, a few cerebellar circumvolutions) are included in the commercial biochips, often excluding deep cerebellar nuclei or white matter, with variability across kits. This might explain the failure in detecting Ma2 antibodies, which typically react with neurons of the deep cerebellar nuclei, or CV2 antibodies, which react with a subpopulation of oligodendrocytes within the white matter.[14]

Another problem when using these commercial IIF-TBAs is that antibody-specific patterns are not recognizable in 31%–

Figure 7 Main Nonspecific Immunostainings Identified in Control (Negative) Samples That May Resemble the Patterns From Positive Samples



Neurofilament-like pattern (A) with diffuse staining of axons in the granular layer and in the white matter of the cerebellum; blood vessels staining (B) with diffuse staining of blood vessels' wall (white arrows); SOX1-like pattern (C) resembling the staining of nuclei of Bergmann glia cells in the Purkinje cell layer of the cerebellum (white arrows); and amphiphysin-like staining (D) with diffuse neuropil staining of the molecular layer. GL = granular layer; ML = molecular layer; PC = Purkinje cell; WM = white matter. Scale bar = 100 μm.

38% of the positive samples and, even when identified, might be misclassified as positive for a wrong antibody (around 7% of cases). This particularly affects the detection of amphiphysin antibodies, which in half of the samples were misclassified as GAD65 antibodies by the INOVA IIF-TBA, and Tr antibodies, which in 30% of cases were misclassified as Yo or GFAP antibodies by the EUROIMMUN IIF-TBA.

Considering that each autoimmune/paraneoplastic antibody occurs in association with a distinct clinical syndrome, tumor type, and demographics (sex, age), misclassification might lead to discordance between antibody and clinical features, resulting in misdiagnosis, delayed treatment, and incorrect cancer screening/monitoring. For example, in a patient with stiff-person syndrome, further diagnostic interventions and follow-up vary substantially depending on whether the patient has amphiphysin or GAD65 antibodies. If amphiphysin antibodies are suggested (based on INOVA IIF-TBA results), a comprehensive tumor screening should be performed and, if negative, repeated every 6 months[1] because amphiphysin antibodies cause high risk of cancer (>70% cases). Conversely, repeated cancer screening is not needed in patients with GAD65 antibody–associated stiff-person syndrome because these antibodies cause low risk of an underlying cancer.[1]

Finally, the frequent nonspecific patterns that occurred in up to 26% of control samples (without autoimmune paraneoplastic syndromes) can lead to unnecessary confirmatory tests (body CT/PET scans and other cancer screening tests, spinal tap, brain MRI) with consequent higher health-related costs and risk of delayed or wrong diagnosis. In addition, immunostaining patterns that resemble antibody-specific patterns, such as neurofilament/GFAP-like or Hu/Ri-like staining, might result in increased rates of GFAP or Hu/Ri antibody misdiagnosis (representing each 37% of the false-positive results). This is important when considering that GFAP and Hu antibodies are among the most frequent antibodies in patients with autoimmune/paraneoplastic neurologic disorders.[15] Moreover, although experienced raters recognized these patterns as "nonspecific," resulting in a low/moderate number of false-positive results (4%–12%), less experienced raters were more likely to misinterpret these results, explaining the higher false-positive rates (up to 22%).

Of note, these and other nonspecific patterns (such as ANA staining, which was excluded in our study) are similar to those reported in several studies, suggesting the presence of autoantibodies/autoimmunity against brain antigens in a variety of neurologic and psychiatric disorders (dementia, schizophrenia, personality and affective disorders, stroke,

Parkinson disease, amyotrophic lateral sclerosis, COVID-19).[16-19] Many of these reports have found these patterns using commercial TBAs with patients' serum, which substantially increases nonspecific tissue staining, and rarely provided the molecular identity of the neuronal target (described as "autoimmune reactivity against undetermined brain epitopes"). In these studies, the reported seroprevalence of those patterns is comparable in healthy and ill individuals[16] who almost never have the expected syndromes. For example, among patients with dementia, the clinical and biological features (CSF markers of neurodegeneration), MRI findings, epidemiologic factors, and comorbidities are similar between those with nonspecific brain immunostaining patterns and seronegative patients, raising question about the pathologic/clinical significance.[17]

Our study has limitations: first, it was performed in a single laboratory, which might limit generalization of our findings to other laboratory settings; second, we only used the dilution recommended by the manufacturer; third, we included the same number of samples for each antibody (n = 10), which does not take into consideration the different prevalence of each antibody. For example, because Hu antibodies are the most common antibodies and EUROIMMUN IIF-TBA allowed the correct identification of 10 of 10 Hu-positive samples, the overall sensitivity of this test might increase in the setting of general laboratory routine. On the contrary, the sensitivity and specificity of both commercial tests may have been overestimated because we used positive samples associated with well-defined syndromes, instead of real-life laboratory workup, which often includes samples from patients with clinical features that have lower pretest probability. Finally, because we included a lower number of negative than positive samples, we assessed more reliably sensitivity than specificity.

The strengths of our study include the large number of antibody-positive and antibody-negative samples, the blinded evaluation of samples by 2 experts with extensive experience in brain immunohistochemistry, and the assessment of these TBAs also by "less experienced" raters.

The main clinical implications are that using commercial IIF-TBAs for IC-Abs detection results in a high rate of false negatives and, particularly with less experienced raters, a substantial rate of false positives. Specific nationwide or international trainings are needed to obtain standardized readouts and improve the diagnostic accuracy of these commercial kits, which are widely used by clinical laboratories.

Owing to their low sensitivity, commercial IIF-TBAs alone should not be used as a screening test because 25%–35% of patients with positive antibodies may be missed. Therefore, if a paraneoplastic neurologic syndrome is clinically suspected, we recommend using a commercial IIF-TBA alongside a more sensitive technique, such as line blots or CBAs. We believe that commercial companies should include in their datasheets a warning about the limitations of these tests when used alone and recommend a double-technique approach. Given that line blots and CBAs have their own limitations (previously reported, but not examined here[3,9]), discordant results between 2 techniques should prompt retesting in a specialized center with in-house TBAs.

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Author Contributions

C. Milano: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data. P. Businaro: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data. C. Papi: drafting/revision of the manuscript for content, including medical writing for content. L. Arlettaz: drafting/revision of the manuscript for content, including medical writing for content. L. Marmolejo: drafting/revision of the manuscript for content, including medical writing for content. L. Naranjo: drafting/revision of the manuscript for content, including medical writing for content. M. Gastaldi: drafting/revision of the manuscript for content, including medical writing for content. R. Iorio: drafting/revision of the manuscript for content, including medical writing for content. A. Saiz: drafting/revision of the manuscript for content, including medical writing for content. J. Planagumà: drafting/revision of the manuscript for content, including medical writing for content. E. Aguilar: drafting/revision of the manuscript for content, including medical writing for content. C. Pizzanelli: drafting/revision of the manuscript for content, including medical writing for content. E. Martinez-Hernandez: drafting/revision of the manuscript for content, including medical writing for content. T. Armangue: drafting/revision of the manuscript for content, including medical writing for content. M. Guasp: drafting/revision of the manuscript for content, including medical writing for content. R. Ruiz-García: drafting/revision of the manuscript for content, including medical writing for content. L. Sabater: drafting/revision of the manuscript for content, including medical writing for content. J.O. Dalmau: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. F. Graus: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. M. Spatola: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data.

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