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Low Prevalence and Inconsistency of LRP4-IgG Detection in Suspected Myasthenia Gravis

A Multicenter CBA Comparison

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Abstract

Background and Objectives

Myasthenia gravis (MG) is an autoimmune disorder primarily caused by antibodies targeting neuromuscular junction proteins, particularly the acetylcholine receptor and the muscle-specific tyrosine kinase. However, 10%–20% of patients with MG are double-seronegative (dsMG). Antibodies against low-density lipoprotein receptor-related protein 4 (LRP4-IgG) have been found in a variable proportion of dsMG cases, but their frequency and clinical relevance remain unclear because of differences in assay methodologies and study populations. In this study, we assessed the frequency of LRP4-IgG in patients with suspected MG using different cell-based assay (CBA) protocols.

Methods

In this multicenter observational study, we enrolled consecutive patients presenting with symptoms suggestive of MG. LRP4-IgG was tested by 2 centers using 3 different CBAs: live (l-CBA), methanol-fixed (mf-CBA), and paraformaldehyde-fixed (pf-CBA). Patients were classified as having MG or other disorders (ODs). Positive samples were cross-tested between centers.

Results

Among 684 patients (302 with MG, 382 with ODs), LRP4-IgG was detected by mf-CBA in 2% (6/302) of MG cases and in 0.52% (2/382) of OD cases. Among patients with MG, 3.9% (4/102) of dsMG and 1% (2/200) of seropositive patients tested positive. Only 50% of mf-CBA–positive cases were confirmed by pf-CBA, and none were detected by l-CBA. Cross-center testing showed partial reproducibility.

Discussion

LRP4-IgG detection in suspected MG is rare and inconsistent across assays, suggesting that routine testing is not currently warranted and that these antibodies are at present of limited diagnostic value. These findings highlight the need for standardized, validated LRP4-IgG assays. Future studies should focus on direct comparison and harmonization of testing protocols to clarify the clinical utility of LRP4-IgG testing.

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Supplementary Material

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Glossary

1-CBA = live CBA; **AChR** = acetylcholine receptor; **CBA** = cell-based assay; **dsMG** = double-seronegative; **GMG** = generalized MG; **LRP4** = low-density lipoprotein receptor-related protein; **mf-CBA** = methanol-fixed CBA; **MG** = myasthenia gravis; **MuSK** = muscle-specific tyrosine kinase; **ODs** = other disorders; **OMG** = ocular myasthenia gravis; **pf-CBA** = paraformaldehyde-fixed CBA; **RT** = room temperature.

Introduction

Myasthenia gravis (MG) is a rare autoimmune disorder characterized by muscle weakness and fatigability, caused by autoantibodies against neuromuscular junction proteins.[1-3] Autoantibodies against acetylcholine receptor (AChR-Ab) and muscle-specific tyrosine kinase (MuSK-Ab) are detected in approximately 80%–85% and 5%–10% of patients with MG, respectively.[4-8] However, around 10%–20% of patients with MG are classified as double-seronegative (dsMG), as they exhibit clinical and electrophysiologic features typical of MG without detectable antibodies against AChR or MuSK.[9-11] The clinical management of these patients remains challenging, highlighting the need for other biomarkers in this subgroup.

Low-density lipoprotein receptor-related protein 4 (LRP4), a member of the LDLR family, has emerged as a potential antigenic target.[12-15] Reported frequencies of LRP4 antibodies (LRP4-IgG) vary widely depending on the assay methodology, the population analyzed, and its ethnic background.[16-22] The cell-based assay (CBA), an immunologic technique that uses live or fixed cells transiently transfected to express high levels of the protein of interest, is widely used to detect autoantibodies in serum or CSF. Different studies reported frequencies of LRP4 antibodies ranging from 0% to 53.84% using different CBA protocols.[16-18,22] Similarly, the frequencies found by enzyme-linked immunosorbent assays (ELISAs) varied from 9.2% to approximately 15%.[20,21] A study using a luciferase-reporter immunoprecipitation assay found that approximately 3% of 300 Japanese patients with MG who were AChR-Ab–negative tested positive for LRP4-Ab[19] (eTable 1). Overall, these studies highlight the variability and uncertainty regarding the prevalence and clinical significance of LRP4 antibodies, which may stem from the heterogeneity of the assay protocols and studied populations.

In this study, we investigated the frequency of LRP4 antibodies in patients with suspected MG recruited from multiple Italian centers using and comparing the 3 different CBA protocols most commonly described in the literature for LRP4 antibody detection: live CBA (l-CBA), fixed CBA with paraformaldehyde (pf-CBA), and fixed CBA with methanol (mf-CBA).

Methods

Patient Enrollment and Sample Collection

Consecutive patients with suspected MG were recruited from 5 Italian centers. Samples from 4 Bologna-based centers were tested at the IRCCS Istituto delle Scienze Neurologiche di Bologna (Center 1). The remaining samples were collected and tested at the Fondazione IRCCS Istituto Neurologico Nazionale Mondino (Center 2).

Comprehensive clinical data were systematically collected at each site and entered into an electronic database. All patients enrolled in the study were tested for AChR and MuSK antibodies using 1-CBA, as previously described[23-25] blindly in respect to the final diagnosis. The final diagnosis was based on clinical presentation and examination, electrophysiologic findings, and response to oral steroids or acetylcholinesterase inhibitor therapy,[2] as well as on the exclusion of other diagnoses. Patients were, therefore, categorized as having MG or other disorders (ODs).

Patients were subsequently classified as seronegative MG if they were negative for AChR and MuSK antibodies by l-CBA and met at least one of the following criteria, in addition to a clinical picture compatible with MG: (1) neurophysiologic confirmation (significant decrement at repetitive nerve stimulation (RNS) or increased jitter at single-fiber EMG (SFEMG); (2) response to pyridostigmine; (3) response to corticosteroid therapy.

CBA for the Detection of LRP4 Antibodies

To detect LRP4 antibodies, 3 distinct CBA protocols were established and conducted at the 2 testing centers. In brief, HEK293T cells were seeded on 12-mm coverslips (Knittel Glass #KN00010062283) and transfected 24 hours (h) after seeding with either the pCMV6-AC-GFP-LRP4 plasmid (OriGene #RG217609) at Center 1 or a pcDNA3.1-Hygro plasmid expressing full-length LRP4 (kindly provided by Dr. Yin Dong [University of Oxford]) at Center 2. For 1-CBA (l-CBA), 24 hours after transfection, cells were incubated with patient serum samples diluted 1:20 in DMEM-HEPES-BSA 1% for 1 hour at room temperature (RT). Cells were then washed 3 times with phosphate-buffered saline (PBS) 1X and fixed with 4% paraformaldehyde (PFA) for 10 minutes. After fixation, 3 washes were performed, followed by incubation with a secondary antibody (Alexa Fluor 594 AffiniPure Fab Fragment Goat Anti-Human IgG, Fcγ fragment specific, Jackson Immune research #109-587-008) diluted 1:1000 for 1 hour. For fixed CBA with paraformaldehyde (pf-CBA), 24 hours after transfection, HEK293T were washed 3 times and fixed with 4% PFA for 10 minutes. After 3 washes, cells were permeabilized with PBS containing 0.2% Triton X-100-bovine serum albumin (BSA) 1% for 5 minutes and then

blocked in PBS-BSA 5% for 1 hour at RT. After blocking, patient serum samples were incubated with cells at a 1:20 dilution in PBS-BSA 5% for 45 minutes. For LRP4 antibody detection, 3 washing steps were performed before adding secondary antibodies as described above. For methanol-fixed CBA (mf-CBA), cells were fixed with ice-cold 100% methanol for 10 minutes, 24 hours after transfection. After 3 washings, cells were incubated with NH_4Cl 50 nM in PBS 1X for 10 minutes, as previously reported.[26] Cells were then washed 3 times with PBS 1X and subsequently blocked with PBS-BSA 5% for 1 hour. Patient serum samples diluted 1:100 were then incubated with transfected cells overnight at 4°C. The following day, after 3 washes, cells were incubated with anti-human secondary antibodies for LRP4-IgG detection. A mouse LRP4 monoclonal antibody (Invitrogen #MA5-27675) served as a positive control for all CBA protocols, used at a dilution of 1:50 for l-CBA and 1:750 for both pf-CBA and mf-CBA. For its detection, Cy3 AffiniPure Donkey Anti-Mouse IgG (H + L) (Jackson Immuno Research #715-165-151) was used as the secondary antibody (1:1000).

Fluorescence microscopy analysis was independently performed by 2 operators who were blinded to each other's results and to the patients' clinical data. Binding was evaluated using a semi-quantitative scoring method ranging from 0 to 4 (0: no signal, 0.5: ambiguous, 1: weak, 2: moderate, 3: strong, 4: very strong signal).[27] Cutoff for positivity was set at 1. Serum samples scored as ambiguous (0.5) or positive (≥1) were re-tested at least twice. Co-localization experiments were also performed to confirm antibody specificity in these cases: transfected cells were incubated with patient serum samples and the relative secondary antibody (Goat Anti-Human IgG, Fcγ fragment specific Alexa Fluor 594). The commercial LRP4 monoclonal antibody and its corresponding secondary antibody (Donkey Anti-Mouse IgG H + L Alexa Fluor 647) were then added to assess co-localization of the staining pattern.

Serum samples confirmed as positive were exchanged between the 2 analyzing centers to evaluate the intercenter agreement.

Statistical Analysis

Continuous and categorical data were reported as means (SD) or percentages, as appropriate. The clinical and demographic features of patients tested by the 2 centers were compared by the Mann-Whitney (continuous variables) and χ^2 (categorical variables) test. All statistical analyses were performed using SPSS version 25, and a p value <0.05 was considered statistically significant. Figures were produced using GraphPad Prism version 8 and BioRender.com.

Standard Protocol Approvals, Registrations, and Patient Consents

All patients provided written consent to the use of their serum samples (taken for diagnostic purposes) in this study. The study was conducted according to the Helsinki Declaration and approved by the Ethical Committee Area Vasta Ethical Committee (project codes CE259-2020-OSS-AUSLBO and CE23070 378-2023-OSS-AUSLBO) and IRCCS San Matteo (project code 0020308/23).

Data Availability

The datasets generated and/or analyzed during this study are available from the corresponding author on reasonable request.

Results

Cohort Features

The cohort included 684 patients: 302 with MG and 382 with OD. Clinical and demographic features of patients tested at each center are summarized in Table 1. Patients' demographic features were similar across the 2 centers, although MG cases were more frequent in Cohort 1 (52.7% vs 31%, $p < 0.0001$, χ^2 test). Differences were also observed in the distribution of ocular myasthenia gravis (OMG) and generalized MG (GMG) between the cohorts ($p = 0.0347$, χ^2 test), with Cohort 1 showing a higher percentage of OMG cases (41.7% vs 29%) and a lower percentage of GMG cases (58.25% vs 71%) compared with Cohort 2. There were no significant differences between the 2 cohorts regarding the percentages of seropositive/seronegative cases, the distribution of AChR/MuSK-positive cases, the incidence of thymoma/thymic hyperplasia, or the proportion of patients with seronegative MG with electrophysiologic confirmation.

LRP4 Antibody Detection and Clinical Features

To investigate LRP4 antibodies, we established 3 distinct CBA protocols for LRP4 expression in HEK293T cells. The successful expression of the protein was confirmed in all protocols using a commercial monoclonal antibody targeting the extracellular domain of LRP4 (Figure 1).

Using the mf-CBA, LRP4-IgG was detected in 8 of 684 patients (1.16%), including 6 from Center 1 and 2 from Center 2 (Figure 2). These findings were corroborated by co-localization experiments in all cases (Figure 3A). However, only 50% of these results were confirmed by pf-CBA (Figure 2). Among the confirmed LRP4-IgG–positive patients, 2 were diagnosed with MG or suspected MG, while the remaining 2 patients received alternative diagnoses, namely motor neuron disease and stroke. Of interest, all patients whose mf-CBA results were not confirmed by pf-CBA were diagnosed with MG. Specifically, 3 of these 4 patients were classified as having OMG, with only 1 testing positive also for clustered AChR antibodies. The remaining patient was an AChR-positive GMG case. Notably, none of the LRP4-Ab–positive samples identified by mf-CBA or pf-CBA tested ambiguous or positive in the l-CBA. The clinical characteristics of the LRP4-IgG–positive patients are presented in Table 2.

Overall, LRP4-IgG was identified in 6 of 302 patients with MG (2%) and in 2 of 382 patients with other diagnoses (0.52%). Among patients with MG, LRP4-IgG was detected

Table 1 Clinical and Demographic Features of Patients Recruited at the 2 Centers

	Cohort 1	Cohort 2	*p* Value
Number of patients	413	271	
M: F	189: 224	126: 145	NS
Age (mean ± SD)	62 ± 19	63.91 ± 18.13	NS
Final diagnosis			
Other diagnoses, n (%)	195 (47.21)	187 (69)	$p < 0.0001$
Myasthenia, n (%)	218 (52.7)	84 (31)	
OMG, n (%)	91 (41.7)	24 (29)	$p = 0.0347$
GMG, n (%)	127 (58.25)	60 (71)	
Seropositive cases, n (%)	146 (67)	54 (64)	NS
AChR positive, n (%)	135 (62)	48 (57)	NS
MuSK positive, n (%)	11 (5)	6 (7)	NS
Seronegative, n (%)	72 (33)[a]	30 (36)[a]	NS
Electrophysiology-confirmed seronegative, n (%)	47 (65.2)	20 (66)	NS
Thymoma, n (%)	32 (14.67)	9 (11)	NS
Thymic hyperplasia, n (%)	20 (9.17)	4 (5)	NS

Abbreviations: GMG = generalized myasthenia gravis; NS = not significant; OMG = ocular myasthenia gravis.
[a] Thirteen patients with seronegative OMG in Cohort 1 and 1 in Cohort 2 showed a modest or incomplete response to AChE inhibitors and were diagnosed on the basis of the clinical features, absence of alternative diagnoses, and response to corticosteroid therapy.

in 2 of 200 seropositive cases (1%) and in 3.9% (4/102) of seronegative MG cases. The distribution of LRP4-IgG positivity across the 2 cohorts is shown in Figure 3B. In the overall group of patients with MG, the positivity rate was 1.83% (4/218) at Center 1 and 2.38% (2/84) at Center 2. Among patients with seronegative MG, LRP4-IgG was found in 2.77% (2/72) of cases at Center 1 and 6.66% (2/30) at Center 2. No patients with seropositive MG or those with other diagnoses showed binding against LRP4 at Center 2. At Center 1, LRP4-IgG was also detected in 1.36% (2/146) of patients with seropositive MG and 1% (2/195) of patients with other diagnoses. Stratifying patients by clinical phenotype, 1 of 187 patients with GMG (0.53%) was LRP4-IgG positive vs 5 of 115 patients with OMG (4.3%) ($p = 0.03$) (Figure 3C). These included 1 of 57 patients (1.8%) who were seropositive for AChR antibodies and 4 of 58 (6.9%) who were double-seronegative.

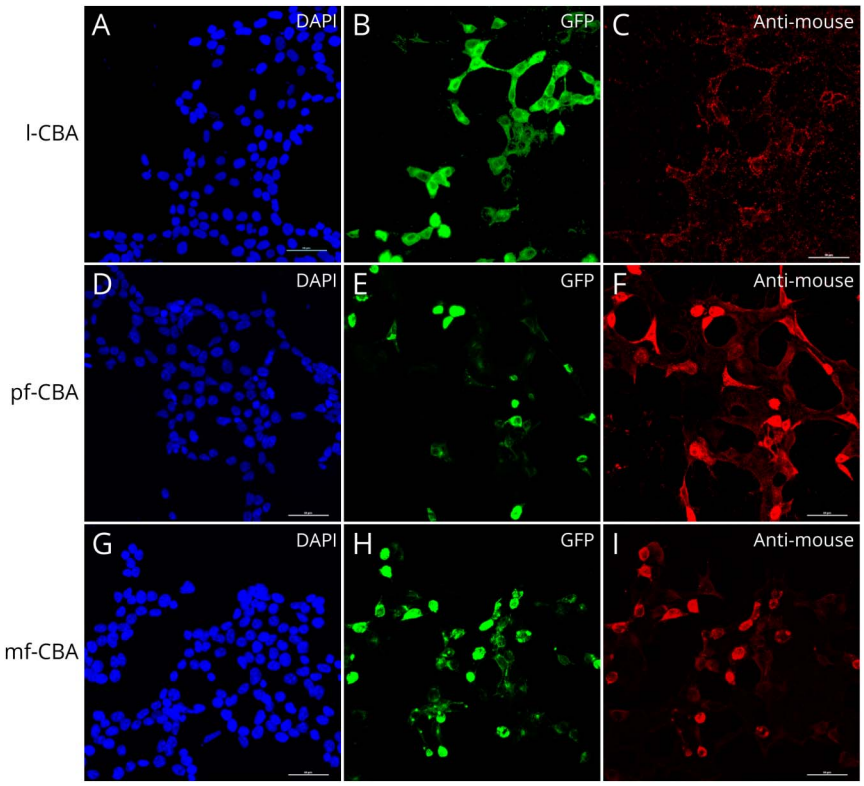
To validate these findings, Center 1 and Center 2 exchanged and cross-tested their positive samples (Figure 4A). As shown in Figure 4B, the results were only partially concordant, underscoring the variability and challenges in LRP4-IgG detection.

Discussion

Over the past decades, LRP4 has emerged as a novel autoantigen in patients with dsMG. In this large multicenter study, among 684 patients with suspected MG, LRP4-IgG was detected in 1.16% of cases using the mf-CBA. Co-localization experiments confirmed these findings. However, only 50% of these positive samples were confirmed using the pf-CBA, and none showed reactivity with l-CBA. Among MG cases, 2% tested positive overall. However, LRP4-IgG was also detected in 0.52% of patients with other neurologic disorders.

Previous studies have reported widely varying frequencies of LRP4 antibodies, particularly among patients with dsMG. Studies using mf-CBA protocols have reported the highest detection rates, up to 33% in patients with MG.[26,28] However, these antibodies were also found by the same group in up to 23% patients with amyotrophic lateral sclerosis (ALS),[29] raising questions about their specificity. Notably, using this protocol, we found an overall frequency of approximately 4% in dsMG, much lower than the previously reported frequency of these antibodies in the Italian population, which ranged from 18.7% to 21.2%.[26,28] We also observed LRP4-IgG in 1% of AChR-seropositive MG cases and none of the MuSK-positive patients, far below the 13% seropositive rate reported in both AChR and MuSK-seropositive cases in prior Italian studies.[28] When using the pf-CBA, we observed a seropositivity rate of 1.96% in patients with dsMG, with no antibodies detected in the seropositive MG population. This is consistent with other studies using the same protocol. For instance, a 5%[30] positive rate was reported in a cohort of 53 patients with dsMG. Comparable results were obtained in a separate study involving

Figure 1 Staining of HEK293 Cells Transfected With LRP4-GFP and Incubated With Commercial Mouse LRP4 Monoclonal Antibody



Three protocols of CBA were performed: l-CBA (A–C), pf-CBA (D–F), and mf-CBA (G–I). 4′,6-diamidino-2-phenylindole (DAPI) (blue channel) shows nuclear staining, and green fluorescent protein (GFP) expression (green channel) indicates transfected cells. For each protocol, LRP4 antibody was visualized with Cy3 anti-mouse IgG (red fluorescence). CBA = cell-based assay; LRP4-IgG = lipoprotein receptor-related protein 4; mf-CBA = methanol-fixed CBA; pf-CBA = paraformaldehyde-fixed CBA.

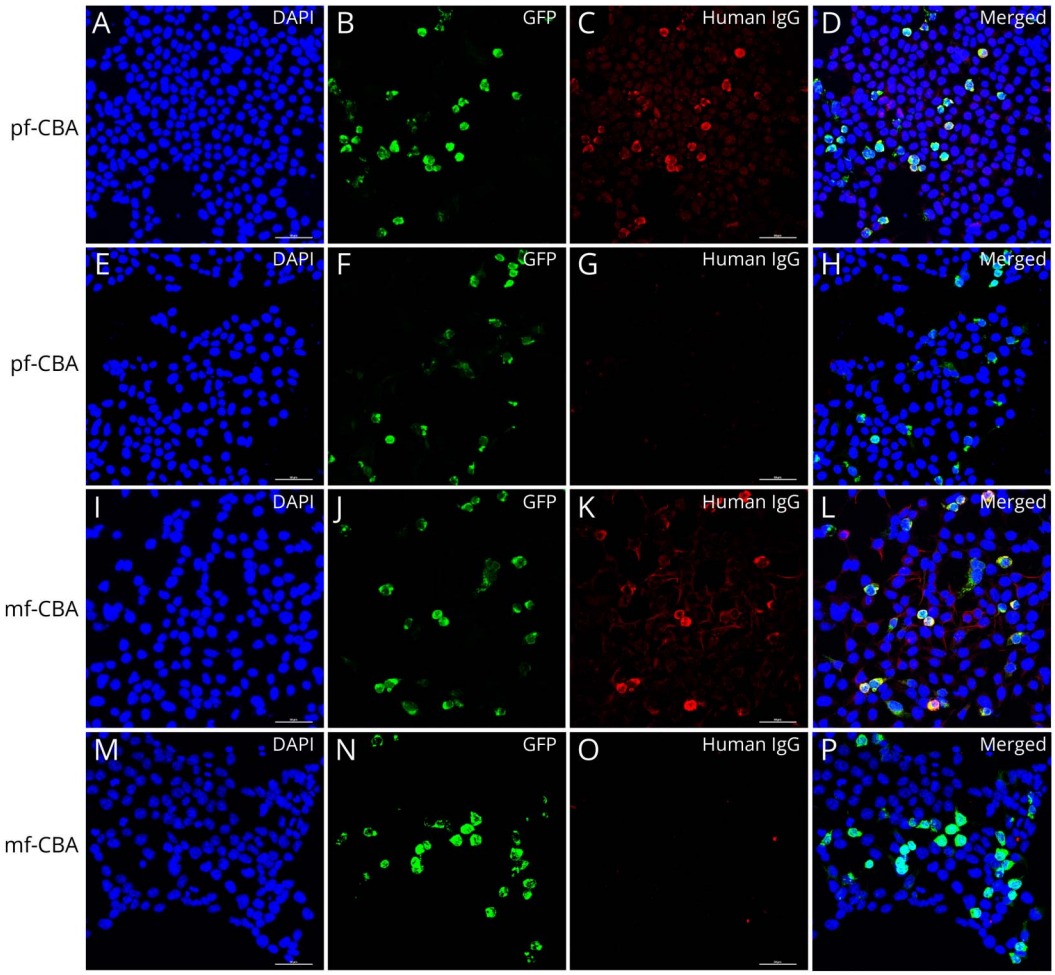
a Chinese population.[16] By contrast, using a commercial CBA, no LRP4-IgG–positive cases were identified among patients with dsMG, while some non-MG patients tested positive[18]. In our study as well, using either fixed CBA protocol, we found 2 LRP4-IgG–positive cases among patients with alternative diagnoses, including motor neuron disease and stroke. Our data, together with other reports from clinical diagnostic services,[18,31] highlight the potential for false positives and raise questions about the specificity of currently available LRP4-IgG testing and overall diagnostic utility for MG.[32,33]

Of interest, none of the LRP4-positive cases in our study were confirmed using the l-CBA. Although we cannot exclude the possibility of a suboptimal surface expression of the protein, our results align with previous studies. Notably, a modified CBA with co-expression of LRP4 and LRP1 (a chaperone protein) to enhance cell surface expression found no LRP4 antibodies in a dsMG cohort,[34] suggesting a low frequency of these antibodies in patients with MG, regardless of membrane expression optimization.

In this regard, a key factor influencing LRP4-IgG detection could be the type of fixation used. Methanol, a coagulating fixative, induces protein precipitation through denaturation and cellular dehydration, without forming covalent bonds, compromising the morphologic and antigenic integrity of the cells.[35-37] This may artificially expose intracellular or denatured epitopes, potentially inflating false-positive rates. By contrast, paraformaldehyde, a cross-linking fixative, better preserves native protein conformation. The low agreement between mf-CBA and pf-CBA in our study supports this concern. Moreover, the absence of positive results in l-CBA, where protein conformation is closest to the native state, reinforces doubts about the specificity of LRP4-IgG signals seen with fixed CBAs.

An additional issue is the reproducibility of LRP4-IgG detection across laboratories. Cross-testing between our 2 centers, both with large experience in CBA testing, showed only partial concordance. While none of the serum samples were positive with the l-CBA in both centers, so that the concordance in this case was total, the reproducibility of fixed-CBAs results was only partial. We can assume that, although some minimal discrepancy might be related to interindividual variations, the positive and negative results are mostly related to interassay differences, including the use of different plasmids. In addition, issues in test interpretation (e.g., in microscopic evaluation) should be taken into account. This emphasizes the urgent need for standardization of LRP4 antibody assays and improved interlaboratory validation before this marker can be reliably used in routine clinical practice.

Figure 2 Fixed CBAs Displaying Representative Examples of Patient Serum Samples Testing Positive and Negative for LRP4 Antibodies



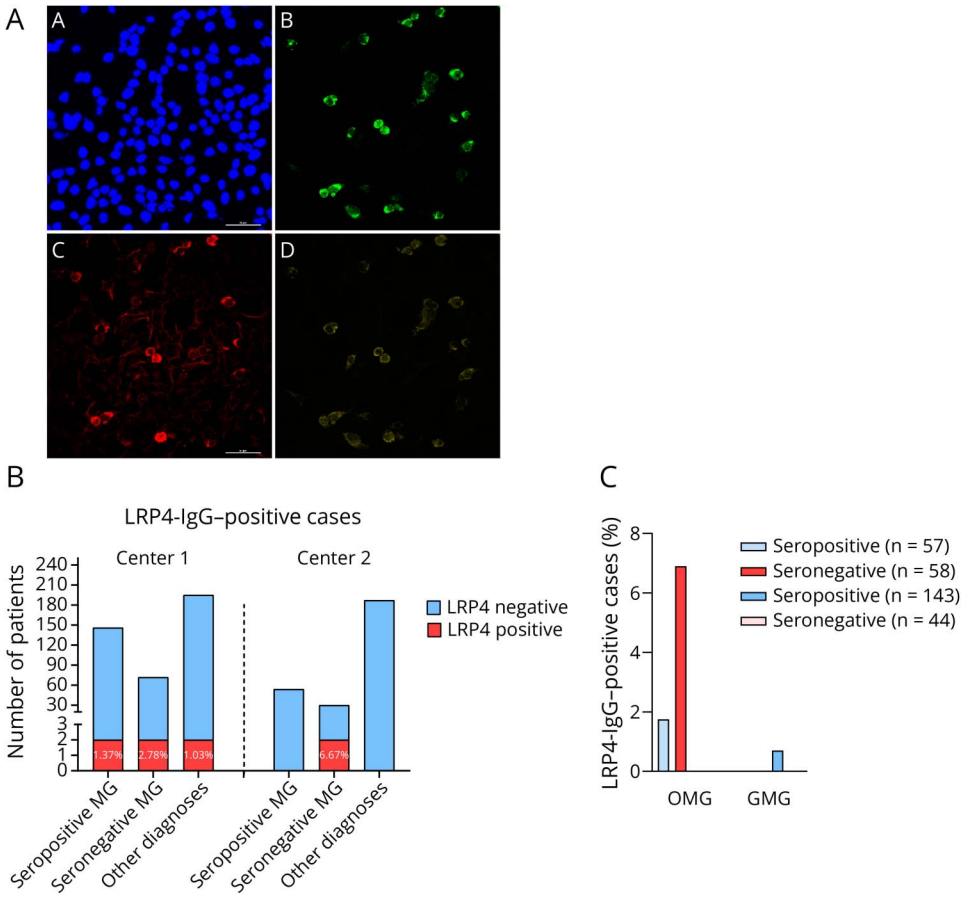
HEK293 cells were transfected with LRP4-GFP and incubated with patient serum samples. The first 2 rows illustrate the pf-CBA, with one patient testing positive (A–D) and the other negative (E–H) for LRP4 antibody detection. The remaining 2 rows display the results of mf-CBA, in which one patient tested positive (I–L) and the other tested negative (M–P), respectively. DAPI (blue channel) shows nuclear staining, and GFP expression (green channel) indicates transfected cells. Human anti-LRP4 antibodies were visualized with anti-human IgG Alexa Fluor 594 (red fluorescence). The last column indicates the merge of all channels. CBA = cell-based assay; LRP4 = lipoprotein receptor-related protein 4; mf-CBA = methanol-fixed CBA; pf-CBA = paraformaldehyde-fixed CBA.

Our study has limitations. The 2 patient cohorts differed significantly regarding MG prevalence, with Center 1 having a higher proportion of MG diagnoses. There were also differences in the proportions of ocular vs generalized cases. These disparities could introduce selection bias or affect assay sensitivity. However, we tested a large population of patients with suspected MG, reflecting a real-world cohort.

Despite these limitations, our findings align with previous studies indicating that antibodies to LRP4 are rare and of uncertain diagnostic relevance. Although potential pathogenic effects of these antibodies have been shown in experimental models,[19,38] one using an in vitro model of agrin-LRP4-MuSK signaling and another using an in vivo active immunization approach, neither study definitely established the pathogenic potential of LRP4 antibodies through passive transfer of human LRP4-IgG. Accordingly, patients with LRP4-IgG generally present with mild symptoms, lack a distinctive clinical phenotype compared with patients with AChR-IgG MG, and respond well to conventional therapies. This suggests that some of these LRP4-IgG–positive patients might further harbor low-affinity AChR antibodies detectable only by CBA.[39] In line with this, 1 of 5 OMG LRP4-IgG–positive cases in our cohort had clustered AChR antibodies detectable only by 1-CBA.

In conclusion, our study suggests that LRP4 antibodies are rarely detected in patients with MG, including those with dsMG, and may be detected in patients with other neurologic conditions. The observed variability across assay types, along with poor intercenter reproducibility, limits the current diagnostic utility of LRP4-IgG testing and even questions whether LRP4-IgG should be considered as a separate disease group. At present, routine screening for LRP4 antibodies in

Figure 3 Validation Experiments and LRP4-IgG Positive Cases Frequency at Center 1 and Center 2



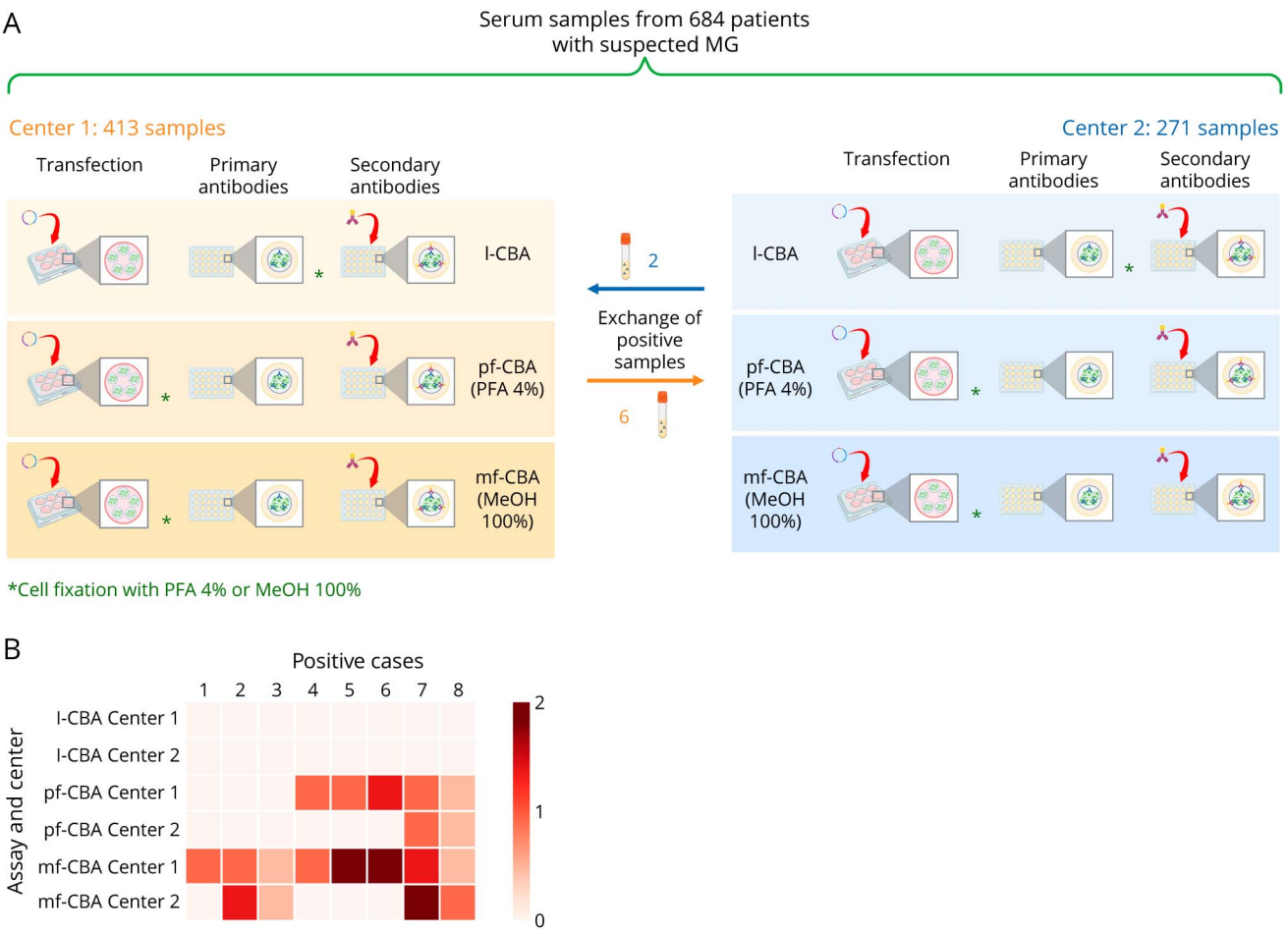
(A) Co-localization experiment using mf-CBA displaying a patient serum sample testing positive for LRP4 antibodies. DAPI (blue) shows nuclear staining, and GFP expression (green) indicates transfected cells. Human anti-LRP4 antibodies were visualized with anti-human IgG Alexa Fluor 594 (red). The commercial LRP4 monoclonal antibody was added and visualized with its corresponding secondary antibody (Donkey anti-mouse IgG H + L Alexa Fluor 647) (yellow). (B) Distribution of positivity across the 2 cohorts and correlation with clinical features and diagnosis. (C) Distribution of LRP4 antibody positivity among patients with OMG and GMG, stratified by seropositive and seronegative status. GMG = generalized myasthenia gravis; LRP4 = lipoprotein receptor-related protein 4; mf-CBA = methanol-fixed CBA; OMG = ocular myasthenia gravis; pf-CBA = paraformaldehyde-fixed CBA.

Table 2 Clinical Features and Laboratory Findings of Patients Testing Positive for LRP4 Antibodies Recruited at Center 1 and Center 2

Patient n	Onset	Ab status (AChR and MuSK)	LRP4 antibody detection protocol	RNS/ SFEMG	Thymic pathology	Final diagnosis	Therapy	Response to therapy
1	Ptosis	Negative	mf-CBA	Negative/ NA	Negative	OMG	Pyridostigmine prednisone	Improvement
2	Ptosis, bulbar symptoms	Positive AChR	mf-CBA	Negative/ NA	Thymoma	GMG	Pyridostigmine prednisone	Improvement
3	Ptosis, diplopia	Positive AChR	mf-CBA	Positive/ positive	Thymic hyperplasia	OMG	Pyridostigmine prednisone	Improvement
4	Fluctuating ptosis	Negative	pf-CBA, mf-CBA	NA/NA	Negative	OMG	Pyridostigmine AZA	Improvement
5	Acute dysphagia	Negative	pf-CBA, mf-CBA	Negative/ NA	NA	Stroke	NA	NA
6	Lower limbs weakness	Negative	pf-CBA, mf-CBA	Negative/ NA	NA	MND	NA	NA
7	Fluctuating diplopia	Negative	pf-CBA, mf-CBA	Negative/ negative	Negative	OMG	Pyridostigmine	Improvement
8	Fluctuating ptosis and diplopia	Negative	mf-CBA	Negative/ negative	Negative	OMG	Prednisone, pyridostigmine	Improvement

Abbreviations: AZA = azathioprine; F = female; GMG = generalized myasthenia gravis; M = male; mf-CBA = methanol-fixed CBA; MND = motor neuron disease; NA = not available; OMG = ocular myasthenia gravis; pf-CBA = paraformaldehyde-fixed CBA; RNS = repetitive nerve stimulation; SFEMG = single-fiber EMG.

Figure 4 Study Design and Heat Map



(A) Study design. Created in BioRender. Serra, L. (2026) BioRender.com/xheohiq. (B) Heat map showing result summary of positive cases and intercenter agreement. Created in BioRender. Serra, L. (2026) BioRender.com/7hpcg9x.

MG is not recommended and LRP4 positivity alone should not be considered sufficient to establish a diagnosis of MG in the absence of compatible clinical and electrophysiologic findings. The development of specific, sensitive, and reproducible assays, ideally validated across centers, will be essential to determine the true diagnostic relevance of LRP4 antibodies in MG.

Author Contributions

I. Gligora: 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. L. Serra: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. S. De Pasqua: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. F. Ricciardiello: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. L. Morelli: major role in the acquisition of data. C. Morandi: major role in the acquisition of data. S. Masciocchi: major role in the acquisition of data. A. Malvaso: major role in the acquisition of data. C. Ballestracci: major role in the acquisition of data. V. Di Stasi: major role in the acquisition of data. V. Vacchiano: major role in the acquisition of data A Fetta: major role in the acquisition of data S Rossi: major role in the acquisition of data. K. Kaveh Moghadam: major role in the acquisition of data. G. Rizzo: major role in the acquisition of data. D.C. Milano: major role in the acquisition of data. A. Zini: major role in the acquisition of data. D. Maria Cordelli: major role in the acquisition of data R Rinaldi: major role in the acquisition of data. V. Donadio: major role in the acquisition of data. R. Liguori: major role in the acquisition of data. M. Gastaldi: drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data. M.P. Giannoccaro: 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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