A multicentre, prospective, double-blind study comparing the accuracy of autoantibody diagnostic assays in myasthenia gravis: the SCREAM study

Zhiguo Li,[a] Chao Zhang,[a] Ting Chang,[b] Xinghu Zhang,[c] Huan Yang,[d] Feng Gao,[e] Jinzhou Feng,[f] Hongbo Liu,[g] Sheng Chen,[h] Lihua Wang,[i] Chunsheng Yang,[a] Huining Li,[a] Yuesong Pan,[c] Jacqueline Palace,[j] and Fu-Dong Shi,[a,c,*] for the SCREAM Study Investigators[k]

[a]Department of Neurology, Tianjin Medical University General Hospital, Tianjin, China
[b]Department of Neurology, Tangdu Hospital, Air Force Medical University, Xi'an, Shanxi, China
[c]Centre for Neurological Diseases, Beijing Tiantan Hospital, Capital Medical University, Beijing, China
[d]Department of Neurology, Xiangya Hospital, Central South University, Changsha, China
[e]Department of Neuroimmunology, Henan Institute of Medical and Pharmaceutical Sciences, Academy of Medical Science, Zhengzhou University, Zhengzhou, China
[f]Department of Neurology, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China
[g]Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China
[h]Department of Neurology, Shanghai Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
[i]Department of Neurology, The Second Affiliated Hospital of Harbin Medical University, Harbin, China
[j]Department of Neurology, John Radcliffe Hospital Oxford, Nuffield Department of Clinical Neurosciences, Oxford University, Oxford, UK

The Lancet Regional Health - Western Pacific 2023;38: 100846

Published Online xxx
https://doi.org/10.1016/j.lanwpc.2023.100846

Summary

Background Laboratory determination of autoantibodies against acetylcholine receptor (AChR), muscle-specific kinase (MuSK) and other autoantigens have been integrated into the diagnosis of myasthenia gravis (MG). However, evidence supporting the selection of methodologies is lacking.

Methods In this prospective, multicentre cohort study, we recruited patients with suspected MG to evaluate the diagnostic accuracy of cell-based assay (CBA), radioimmunoprecipitation assay (RIPA) and enzyme-linked immunosorbent assay (ELISA) in detecting AChR and MuSK autoantibodies. This study is registered with www.clinicaltrials.gov, number NCT05219097.

Findings 2272 eligible participants were recruited, including 2043 MG, 229 non-MG subjects. AChR antibodies were detected in 1478, 1310, and 1280 out of a total of 2043 MG patients by CBA, RIPA, and ELISA, respectively; sensitivity, 72.3% (95% CI, 70.3–74.3), 64.1% (95% CI, 62.0–66.2), 62.7% (95% CI, 60.5–64.8); specificity, 97.8% (95% CI, 95.0–99.3), 97.8% (95% CI, 95.0–99.3), 94.8% (95% CI, 91.9–97.7). MuSK antibodies were found in 59, 50, and 54 from 2043 MG patients by CBA, RIPA and ELISA, respectively; sensitivity, 2.9% (95% CI, 2.2–3.7), 2.4% (95% CI, 1.8–3.2), 2.6% (95% CI, 2.0–3.4); specificity, 100% (95% CI, 98.4–100), 100% (95% CI, 98.4–100), and 99.1% (95% CI, 96.9–99.9). The area under the curve of AChR antibodies tested by CBA was 0.858, and there were statistical differences with RIPA (0.843; $p = 0.03$) and ELISA (0.809; $p < 0.0001$).

Interpretation CBA has a higher diagnostic accuracy compared to RIPA or ELISA in detecting AChR and MuSK autoantibodies for MG diagnosis.

Funding New Terrain Biotechnology, Inc., Tianjin, China.

Keywords: Myasthenia gravis; Autoantibody diagnostic assays; Anti-acetylcholine receptor antibody; Anti-muscle-specific kinase antibody

*Corresponding author. Department of Neurology, Institute of Neuroimmunology, Tianjin Medical University General Hospital, Tianjin, China.
E-mail address: fshi@tmu.edu.cn (F.-D. Shi).
[k]List of the SCREAM Study Investigators is provided in the Supplementary Appendix.

Research in context

Evidence before this study

We queried PubMed and Web of Science for articles published from January 1, 1970 to December 30, 2020, without language restrictions, using the search terms: "cell-based assay (CBA)", "radioimmunoprecipitation assay (RIPA)", "enzyme-linked immunosorbent assay (ELISA)", "acetylcholine receptor (AChR)", "muscle-specific kinase (MuSK)" and "myasthenia gravis (MG)". A total of 16 studies were identified with regard to methodologies in detecting AChR and (or) MuSK antibodies.

All the retrieved studies were retrospective diagnostic studies except for one prospective study. In these studies, the number of enrolled MG patients ranged from 16 to 153 for detection of AChR antibodies, and from 65 to 875 for MuSK antibodies. None of these studies were designed to compare the specificity and sensitivity of CBA, RIPA, and ELISA in the determination of AChR or MuSK antibodies, in parallel. These studies were limited to small sample sizes, single centre or unblinded diagnostic design, resulting in significant variability on the sensitivity and specificity of the assays. In some studies, divergent results were derived from these assays. Thus, the current guidelines for the diagnosis of MG generally lacks evidence-based recommendations on methodology for detecting AChR and MuSK autoantibodies. This may impact the accuracy in clinical decision making across centres and poses a challenge for neurologists managing MG patients and conducting clinical trials.

Added value of this study

This is the first prospective, multicentre, large cohort diagnostic study on the determination of AChR and MuSK antibodies for MG. We recruited 2325 patients with suspected MG and compared the sensitivity and specificity of different assays of CBA, RIPA, and ELISA detection of AChR and MuSK autoantibodies. This study indicates that compared to RIPA and ELISA, CBA increased the absolute yield of detection of AChR or MuSK autoantibodies by 8.2%–9.6% and 0.3%–0.5%, respectively. In addition, CBA has a higher specificity (97.8% for AChR antibody and 100% for Musk antibody, respectively). This study favors CBA over RIPA and ELISA for detecting AChR and MuSK antibodies for the diagnosis of MG.

Implications of all the available evidence

CBA has a higher diagnostic accuracy compared to RIPA or ELISA in detecting AChR and MuSK autoantibodies for MG diagnosis.

Introduction

Myasthenia gravis (MG) is a neuromuscular junction (NMJ) disorder mediated by autoantibodies against the acetylcholine receptor (AChR), muscle-specific kinase (MuSK), and other autoantigens at the postsynaptic membrane of the NMJ.[1–3] AChR and MuSK autoantibodies are serological indicators that establish a definitive diagnosis of MG, fluctuation of these antibodies levels may reflect the disease status and the responsiveness to immune modulatory treatment.[4–10] In the past decades, the diagnostic accuracy of cell-based assay (CBA), radioimmunoprecipitation assay (RIPA) and enzyme-linked immunosorbent assay (ELISA) for AChR and MuSK antibodies has been examined in several studies.[11–18] However, these studies were limited to small sample size, single centre and unblinded diagnostic design. Furthermore, the recent evolution of these assays yield significant variability in terms of their sensitivity and specificity.[11–16] In some cases, contrary conclusions were made regarding the sensitivity of these assays.[17,19] Consequently, there is no uniform consensus regarding the diagnostic method to determine the AChR and MuSK antibodies. The current diagnosis and management guidelines of MG[6,8,20–22] generally lacks evidence-based recommendations on methodology in detecting AChR and MuSK autoantibodies. This can impact the accuracy in clinical decision making across centres, resulting in a challenge for neurologists managing MG patients and conducting clinical trials. To this end, we conducted a multicentre, double-blind, prospective cohort study to compare the sensitivity, specificity, and clinical correlation of CBA, RIPA, and ELISA in detecting AChR and MuSK antibodies in MG diagnosis.

Methods

Study design and participants

In this prospective cohort study, we recruited patients suspected with MG between January 1, 2021 and September 30, 2022, from nine MG centres across China. In April 2021, we ceased the enrollment of non-MG patients when the actual enrollment reached 229 non-MG patients (38.2% in total enrolled patients), which is much larger than the estimated sample size of 138 non-MG required for the study. The enrollment of MG patients continued until September 2022 when the number of MG patients reached 2043. Eligibility inclusion criteria were those 1–90 years of age, with clinical symptoms suggestive of MG. The cardinal clinical symptoms suggestive of MG refer to fluctuating and fatigable weakness of voluntary (skeletal) muscles, i.e., ptosis, diplopia, difficulty in speaking or swallowing, or weakness of the limbs. These symptoms could be worsened during continued activity and partially or fully restored after rest. Strength may be improved following the administration of anticholinesterase drugs such as neostigmine. MG diagnosis and differential diagnosis

work up includes serological tests, electrophysiological, pharmacological neostigmine test, thymic computed tomography (CT), and magnetic resonance imaging (MRI). All of these tests, except MRI, were administered to the patients enrolled in this trial to minimize misdiagnosis or to identify clues leading to alternative diagnosis. A fraction of patients had brain stem MRI scanned to rule out brain lesions that caused cranial nerve palsy. Nevertheless, this does not mean all these tests have to be conducted to support the diagnosis in settings of regular patient management. All tests were administered to affirm a definite diagnosis of MG versus non-MG. All patients suspected of MG and found to be consistent with the diagnosis according to the clinical features and non-antibody testing diagnostic work up were enrolled. Data collection were planned before the study were performed.

Exclusion criteria were patients with uncertain diagnoses, incomplete clinical data, or insufficient serum for serological test. The study protocol was approved by the institutional review board and ethics committees at each participating institution. All patients provided written informed consent before study inclusion according to the Declaration of Helsinki.

AChR and MuSK antibody determinations

All patient's clinical data were masked and monitored by members of the ethics committees. At patient enrollment, serum specimens from 2325 participants were collected and renumbered, without any clinical information, and divided into equal aliquots that were randomly allocated to three independent and qualified clinical diagnostic laboratories for AChR and MuSK autoantibodies tests by fixed CBA (Tianjin New Terrain Biotechnology, Inc., Tianjin, China), RIPA (RSR limited, UK) and ELISA (AChR kits from RSR limited, UK; MuSK kits from IBL International, Germany) kits.

In the fixed CBA assay of AChR antibody, 293 T cells were transfected with the α, β, δ, γ and ε subsets of fetal and adult AChR and rapsyn with a ratio of 2:1:1:1:1, respectively. In the MuSK antibody fixed CBA assay, EGFP labeled MuSK were transfected to 293 T cells cultured in vitro. The transfected cells were fixed with 4% polyformaldehyde. After incubating the fixed cells with patient sera, AChR or MuSK antibody of patients binds to AChR or MuSK antigens expressed on the seeded 293 T cells to form a antigen-antibody complex, which can be detected by immunofluorescence labeled anti-human IgG secondary antibody under immunofluorescent microscope. Similarities and differences of these CBA compared with those provided by other vendors such as EUROIMMUN (Product No. FA 1435-1005-2) are provided in the Supplemental Study Protocol version 3.0, page 10. The serum of enrolled patients was diluted at a ratio of 1:10, 1:32, 1:100, 1:320 and 1:1000 for fixed CBA detection in this study. There were 6 titers (<1:10, 1:10, 1:32, 1:100, 1:320 and 1:1000) to estimate the relative intensity of AChR and MuSK antibodies for each sample. Antibody negative in the fixed CBA assay is demarcated as titer <1:10 if no positive signal was detected when serum diluted by 1:10. AChR or MuSK antibody positivity is assigned upon a positive reaction for AChR or MuSK IgG at the titer 1:10. Overall positive results contained samples with weak positivity (titer 1:10 or 1:32), medium positivity (titer 1:100), and strong positivity (titer ≥1:320). For the few inconsistent interpretation results, the sample would be re-tested and re-interpreted to ensure that the interpretation results of the two readers were aligned.

In the RIPA and ELISA assay of AChR and MuSK antibody, the assay principle and procedure were followed by the instruction guideline of these kits.

All specimens of participants were screened with AChR antibody by fixed CBA (AChR antibody CBA kit, recommended cut-off value = 1:10), RIPA (recommended cut-off value = 0.5 nmol/L) and ELISA (recommended cut-off value = 0.45 nmol/L). MuSK antibodies were tested by fixed CBA (recommended cut-off value = 1:10), RIPA (recommended cut-off value = 0.05 nmol/L) and ELISA (recommended cut-off value = 0.4 U/mL). Diagnostic operations, result analyses and quality control were performed according to manufacturer's instructions for respective kits. For each test of each method, stringent quality controls were used to conducted, to ensure the repeatability and accuracy of the research results that were not affected by sites or trials. Each experiment contained positive and negative controls as reference. A positive control of titer 1:10 (the given cut-off value of assay kits) was used in each experiment to minimize the variation between sites or trials. Assay calibrators were also included in RIPA or ELISA assay in which a coefficient of variation less than 15% in intra-or inter assay precision was considered as valid between trials or the sites.

In each assay, there were two statisticians, blinded to the operation of the assay, who independently carried out data analysis. The results were considered valid only if the two interpretations were concordant.

Diagnosis of myasthenia gravis

MG was diagnosed by two consulting neurologists, based on compatible clinical features of skeletal muscles weakness, including ptosis, diplopia, dysphonia, dysphagia, or limb weakness together with one or two of the following criteria: (a) electrophysiological study findings compatible with a postsynaptic neuromuscular junction disorder (repetitive stimulation, single-fiber electromyography, or both); and (b) a response to cholinesterase inhibitors. Electrophysiological testing and neostigmine mesylate tests were operated by different qualified clinical neurologists. Each patient diagnosed with MG was independently diagnosed by

two given qualified neurologists in each centre. These neurologists received assessment with unified diagnostic standards to ensure that their diagnostic consistency reached 98%. In the event of an inconsistent diagnosis, the leading principal investigator in each centre will initiate evaluation by the diagnostic committee to reach final diagnosis. For each sample, diagnostic data were blinded to the operators and readers between different assays or tests. The assays in the diagnosis of MG were independently performed at each centre and their results were provided as clinical data for each enrolled patient. The diagnosis of MG was performed in a double-blinded manner to our investigated tests.

All MG patients participated the study were interviewed to confirm the MG diagnosis during 3-months follow-up after enrollment. Controls were patient who were suspicious for myasthenia but ultimately failed to meet the above standard diagnostic criteria. The disease type of non-MG controls was further defined by other standard diagnostic tests. Patients with uncertain diagnoses were excluded in this cohort.

Statistical analysis

The statistical analysis was performed by independent statistical teams.[23] We calculated that a sample size of 522 (which includes 384 subjects with MG and 138 non-MG) would achieve 90% power to detect a change in sensitivity of 0.5–0.9 by 0.1 under a two-sided binomial test with $\alpha = 0.05$ when the specificity was 0.9–1 in the CBA, RIPA, and ELISA assays.

Based on the given standard diagnosis of myasthenia gravis and the recommended cut-off values of CBA, RIPA and ELISA kits, the sensitivity, specificity, consistency rate, positive likelihood ratio (+LR) and negative likelihood ratio (–LR) with their 95% confidence intervals were respectively calculated for CBA, RIPA, and ELISA. The sensitivity and specificity with their 95% confidence intervals were also calculated for the assays combining two methodologies of CBA, RIPA, and ELISA. To investigate the relationship between clinical features and the consistency of diagnostic methods, sex and age, were used for stratification, sensitivity and specificity were calculated. A Cohen's kappa value was calculated to reflect the consistency between readers in tests.

In the receiver operated characteristic (ROC) curve assay, the sensitivity and specificity of CBA, RIPA, and ELISA on AChR and MuSK antibodies were re-assessed with the raw data. The optimal cut-off values were the concentration of AChR or MuSK antibodies when the investigated assay had the maximum of Youden index (Youden index = sensitivity + specificity–1). Row data were used in the ROC analysis and the Delong test was applied to compare the difference in area under the curve (AUC) values among CBA, RIPA and ELISA.[24] p values of 0.05 or lower are statistically significant.

Indeterminate results were inconsistent positive or negative outcome of a sample in an assay with multiple replicates which often occurs when the results were around the recommended cut-off value from the instructions of each assay. Indeterminate results for AChR and MuSK autoantibodies tests were repeated and participants missing either test were excluded from the analysis. Missing data were categorized into a "missing" category if not available. Some patients were ocular myasthenia gravis (OMG) at the time of blood sample collection, and after a period of time, they became generalized myasthenia gravis (GMG). We kept these patients in OMG group in final data analysis.

All analyses were performed using the statistical software package R4.1.3, and the GraphPad Prism 8 software was used to create graphs.

This trial is registered with www.clinicaltrials.gov, number NCT05219097.

Role of the funding source

The study funder had no role in study design, data collection, data analysis, data interpretation, writing of the report, or decision to submit the results for publication. The corresponding author had full access to all the data in this study and had final responsibility for the decision to submit for publication.

Results

Participants

Between January 1, 2021 and September 30, 2022, 2325 potential eligible participants were enrolled. After excluding 53 (2.3%) ineligible participants (insufficient serum for serological test 12 [0.5%]; incomplete clinical data 41 [1.8%]) (Fig. 1), the final 2272 eligible participants included 2043 MG patients and 229 non-MG patients. The MG patients included 1110 with GMG and 933 with OMG. There were 168 of 229 (73.4%) of non-MG patients that presented with a differential diagnosis including peripheral neuropathy, Guillain–Barré syndrome, Lambert–Eaton myasthenic syndrome (LEMS), amyotrophic lateral sclerosis, external ophthalmoplegia, congenital myasthenia syndrome, cavernous sinus syndrome, etc. 61 non-MG participants were defined as healthy individuals (Supplemental Table S1). Basic clinical characteristics including the age, sex and race/ethnicity of all eligible participants are summarized (Table 1). After the patient was enrolled, MG diagnosis was conducted simultaneously with the collection of serum for MG autoantibody testing. There were no serious adverse events from performing MG diagnosis and serum collection for enrolled patients.

Diagnostic accuracy analysis with the given cut-off values

We first analyzed the diagnostic sensitivity of CBA, RIPA, and ELISA for AChR and MuSK antibodies using

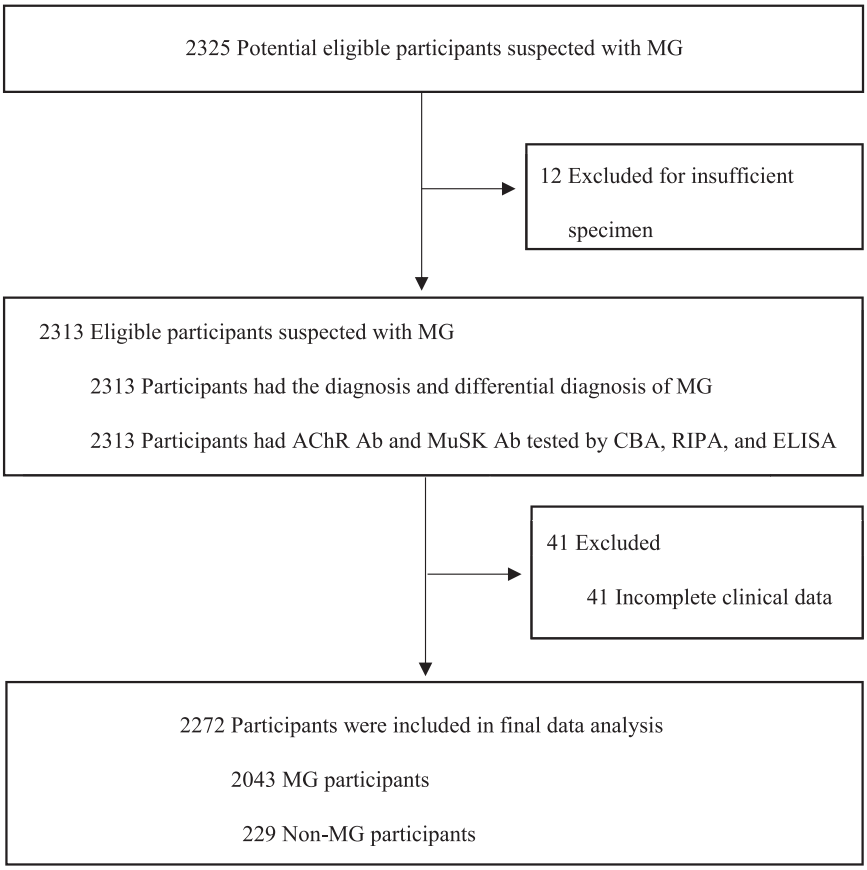

Fig. 1: **Flow of patient enrollment and determination of AChR or MuSK autoantibodies.** The 2325 potential eligible participants were patients that were suspected of myasthenia with need to perform diagnostic evaluation for MG. CBA, RIPA, and ELISA assays were performed blinded to MG diagnosis. MG was diagnosed by two consulting neurologists with the standard diagnosis outlined in the Methods, if patients were ocular myasthenia gravis (OMG) at the time of blood sample collection, and after a period, they became generalized myasthenia gravis (GMG), we retained these patients in OMG group in final data analysis. Controls were patient who were suspicious for myasthenia but ultimately failed to meet the standard diagnostic criteria for MG. The disease type of non-MG controls was further defined by other standard diagnostic tests. Patients with uncertain diagnosis as part of individual with incomplete clinical data were excluded in this cohort. 2043 participants with MG disease and 229 non-MG controls were included in the final data analysis. MG, myasthenia gravis; AChR antibody, acetylcholine receptor antibody; MuSK Ab, muscle-specific kinase antibody; CBA, cell-based assay; ELISA, enzyme-linked immunosorbent assay; RIPA, radioimmunoprecipitation assay; CT/MRI, computed tomography/magnetic resonance imaging.

the recommended cut-off values provided by manufacturers. We used fixed-CBA with AChR or MuSK labeled with EGFP to detect AChR or MuSK IgG of all samples (Supplemental Figs. S1 and S2). Two readers independently evaluated the immunofluorescence of each titer and reported AChR or MuSK antibody signal in each sample and titer; this study achieved a Cohen's kappa value of 0.936 to reflect consistency. Of 2043 MG patients, CBA, RIPA, and ELISA, respectively detected 1478, 1310, and 1280 samples that were positive for AChR antibodies, with sensitivity of 72.3% (95% CI, 70.3–74.3), 64.1% (95% CI, 62.0–66.2), 62.7% (95% CI, 60.5–64.8) (Table 2). In the total 1478 AChR antibody positive samples by CBA, AChR antibody positive rate was 86.3% and 84.6% detected by RIPA and ELISA, respectively. Meanwhile, 6% and 5.8% of total 565 CBA AChR antibody negative samples were also positive in RIPA and ELISA assays (Supplemental Table S2). MuSK antibodies were found in 59, 50, and 54 of 2043 MG patients respectively detected by CBA, RIPA, and ELISA, with corresponding sensitivity of 2.9% (95% CI, 2.2–3.7), 2.4% (95% CI, 1.8–3.2), 2.6% (95% CI, 2.0–3.4). The consistency of CBA, RIPA, and ELISA in detecting MuSK IgG are shown in Supplemental Table S3.

AChR antibody testing by CBA, RIPA, and ELISA respectively found 5, 5, and 12, of 229 control samples to be positive, with the specificity of 97.8% (95% CI,

Participant characteristics	MG (n = 2043)	GMG (n = 1110)	OMG (n = 933)	Control (n = 229)
Age—yr[a]				
Mean	47.1 ± 20.6	50.3 ± 20.3	43.3 ± 22.5	52.6 ± 15.9
Range—No. (%)	0.5–94	0.5–94	0.5–89	10–79
≤20 years	309 (15.1)	117 (10.5)	192 (20.6)	6 (2.6)
21–45 years	516 (25.3)	276 (24.9)	240 (25.7)	66 (28.8)
46–64 years	680 (33.3)	374 (33.7)	306 (32.8)	91 (39.8)
≥65 years	538 (26.3)	343 (30.9)	195 (20.9)	66 (28.8)
Sex—No. (%)				
Male	775 (37.9)	363 (32.7)	412 (44.2)	107 (46.7)
Female	1268 (62.1)	747 (67.3)	521 (55.8)	122 (53.3)
Race or ethnic group—No. (%)				
Asian	2039 (99.8)	1107 (99.7)	932 (99.9)	228 (99.6)
Caucasian	4 (0.2)	3 (0.3)	1 (0.1)	1 (0.4)
Gravis Foundation of America (MGFA)[b]—No. (%)				
I	933 (45.7)	0 (0.0)	933 (100.0)	NA
II	652 (31.9)	652 (58.7)	0 (0.0)	NA
III	283 (13.9)	283 (25.5)	0 (0.0)	NA
IV	152 (7.4)	152 (13.7)	0 (0.0)	NA
V	23 (1.1)	23 (2.1)	0 (0.0)	NA

MG, myasthenia gravis; OMG, ocular myasthenia gravis; GMG, generalized myasthenia gravis. [a]Age of patients at the time of enrollment. [b]MGFA score was performed to estimate the severity of MG when the patient was enrolled.

Table 1: **Baseline characteristics of the participants.**

95.0–99.3), 97.8% (95% CI, 95.0–99.3), 94.8% (95% CI, 91.9–97.7). Out of 229 control samples, 0 MuSK positive cases were detected by CBA or RIPA and 2 positives detected by ELISA with the respective specificity of 100% (95% CI, 98.4–100), 100% (95% CI, 98.4–100), 99.1% (95% CI, 96.9–99.9). These AChR or MuSK antibody positive non-MG samples were from patients with peripheral neuropathy, Guillain–Barré syndrome, external ophthalmoplegia, neuromyelitis optica spectrum disorders (NMOSD), MOG antibody associated disease (MOGAD), and even healthy individuals (Supplemental Table S1 and Supplemental Fig. S3). The detailed methodological differences in AChR and MuSK antibodies are shown in subgroup analysis of MG samples stratified by disease subtype, sex and age (Supplemental Fig. S3; Table 2 and Supplemental Tables S4–S6).

Diagnostic accuracy analysis with the ROC curve

There were 2272 enrolled participants including the 2043 MG patients (ocular myasthenia gravis, 933; generalized myasthenia gravis, 1110) and 229 non-MG individuals were included in the ROC analysis. For the total MG patients, the AUC of AChR antibodies tested by CBA was 0.858 (95% CI, 0.85–0.868), which is higher than that of RIPA (0.843 [95% CI, 0.828–0.858]; p = 0.03) and ELISA (0.809 [95% CI, 0.79–0.826]; p < 0.0001) (Fig. 2A). For the OMG subtype, the AUC of AChR antibodies tested by CBA was 0.802 (95% CI, 0.782–0.82), and there were differences between RIPA (0.785 [95% CI, 0.763–0.81]; p = 0.107) and ELISA (0.736 [95% CI, 0.71–0.762]; p < 0.0001) (Fig. 2B). For the GMG subtype, the AUC of AChR antibodies tested by CBA was 0.904 (95% CI, 0.89–0.91), and there were differences between the RIPA (0.892 [95% CI, 0.877–0.91]; p = 0.0255) and ELISA (0.869 [95% CI, 0.85–0.89]; p < 0.0001) tests (Fig. 2C). In the ROC analysis, the sensitivity, specificity and Youden index of the CBA, RIPA and ELISA are shown in Supplemental Tables S7–S9.

Based on the ROC analysis with the 2043 MG patients and 229 non-MG individuals, the AUC of MuSK antibodies tested by CBA was 0.514 (95% CI, 0.49–0.53) which has no significant difference to that of RIPA (0.513 [95% CI, 0.49–0.53]; p = 0.1572) or ELISA (0.505 [95% CI, 0.48–0.52]; p = 0.062) (Supplemental Fig. S4).

Discussion

In this prospective study evaluating diagnostic methodology for the detection of AChR and MuSK autoantibodies, the CBA assay increased the total absolute yield of AChR or MuSK antibodies in MG patients by 8.2%–9.6% or 0.3%–0.5% compared to that of the RIPA and ELISA tests. Meanwhile, the CBA assay maintained a high diagnostic specificity (97.8% and 100%) compared to that of the RIPA (97.8% and 100%) and ELISA (94.8% and 99.1%). This study provides high-level evidence supporting CBA as a first-line assay in the diagnosis and management of MG as well as evaluating patients with

	MG (n = 2043)		GMG (n = 1110)		OMG (n = 933)		Control (n = 229)	
	TP/(TP + FN)	Se (95% CI)	TP/(TP + FN)	Se (95% CI)	TP/(TP + FN)	Se (95% CI)	TN/(TN + FP)	Sp (95% CI)
AChR Ab								
CBA	1478/2043	72.3 (70.3–74.3)	903/1110	81.4 (78.9–83.6)	575/933	61.6 (58.4–64.8)	224/229	97.8 (95.0–99.3)
RIPA	1310/2043	64.1 (62.0–66.2)	851/1110	76.7 (74.1–79.1)	459/933	49.2 (45.9–52.5)	224/229	97.8 (95.0–99.3)
ELISA	1280/2043	62.7 (60.5–64.8)	829/1110	74.7 (72.0–77.2)	451/933	48.3 (45.1–51.6)	217/229	94.8 (91.9–97.7)
MuSK Ab								
CBA	59/2043	2.9 (2.2–3.7)	44/1110	4.0 (2.9–5.3)	15/933	1.6 (0.9–2.6)	229/229	100 (98.4–100)
RIPA	50/2043	2.4 (1.8–3.2)	41/1110	3.7 (2.7–5.0)	9/933	1.0 (0.4–1.8)	229/229	100 (98.4–100)
ELISA	54/2043	2.6 (2.0–3.4)	44/1110	4.0 (2.9–5.3)	10/933	1.1 (0.5–2.0)	227/229	99.1 (96.9–99.9)
Combined AChR Ab and MuSK Ab[b]								
CBA	1529/2043	74.8 (72.9–76.7)	942/1110	84.9 (82.6–86.9)	587/933	62.9 (59.7–66.0)	224/229	97.8 (95.0–99.3)
RIPA	1358/2043	66.5 (64.4–68.5)	890/1110	80.2 (77.7–82.5)	468/933	50.2 (46.9–53.4)	224/229	97.8 (95.0–99.3)
ELISA	1327/2043	65 (62.8–67.0)	866/1110	78 (75.5–80.4)	461/933	49.4 (46.2–52.7)	215/229	93.9 (90.0–96.6)

AChR Ab, acetylcholine receptor antibody; MuSK Ab, muscle-specific kinase antibody; TP, true positive; FN, false negative; TN, true negative; FP, false positive; Combined AChR and MuSK, AChR IgG$^+$ or MuSK IgG$^+$. [a]The recommended cut-off value of CBA, RIPA, ELISA to AChR antibody was titer 1:10, 0.5 nmol/L and 0.45 nmol/L respectively. The recommended cut-off value of CBA, RIPA, ELISA to MuSK antibody was 1:10, 0.05 nmol/L and 0.4 U/mL respectively. [b]Combined AChR Ab and MuSK Ab means both AChR Ab and MuSK Ab were calculated together when assessing the diagnostic accuracy of CBA, RIPA, and ELISA.

Table 2: **The sensitivity and specificity of CBA, RIPA and ELISA to AChR or MuSK antibody with recommended cut-off value.**[a]

suspected MG for whom AChR or MuSK antibodies are undetectable by RIPA or ELISA assays.

In this study, there is no significant difference of the sensitivity and specificity of MuSK antibodies tested by CBA, RIPA, or ELISA. The presence of MuSK antibodies only accounts for a small fraction (1%–4%) of MG patients.[7] Although we enrolled 2043 MG patients, MuSK antibodies only existed in 50–59 MG patients. This low number of MuSK antibody positive MG may not genuinely reflect the sensitivity of the three tested methods to MuSK antibody. Increasing sample size for this particular group of MG patients is warranted for a definite conclusion on the superiority between the three methods tested. Nevertheless, our results depict an increasing trend in the number of MuSK positivity registered MG by CBA assay (59 for CBA, 50 for RIPA and 54 for ELISA), CBA assay also maintains a high specificity for MuSK antibodies (100%).

Our data indicates that AChR and MuSK antibodies were present in 2.2–5.2% or 0–0.9% of non-MG persons (Supplemental Table S1). These individuals were diagnosed with autoimmune diseases other than MG, extraocular muscle paralysis, as well as a few "healthy individuals" without substantive pathology. In some AChR or MuSK positive non-MG patients, detection errors were unlikely as two or three of these methods resulted in concurrent positive results. As control samples were not pre-selected in our study, the false positive rate may reflect the frequency of AChR and MuSK antibodies in non-MG patients in real-life situation. Nevertheless, non-specific binding and false positivity cannot be entirely excluded, as the specificity of selected methodology may be influenced by systematic errors of the reagent source and corresponding batch effects. Similar observations have been reported in other cohorts[25,26] although the definite explanation for this observation is unavailable at this point. Despite the very low frequency of anti-AChR or MuSK in non-MG individuals, our results reinforce the notion that definite diagnosis of MG requires clinical manifestations in conjunction with a battery of laboratory tests. In addition to superior sensitivity and specificity demonstrated here, CBA assay has a short turnaround time in detecting AChR and MuSK antibodies. In this study, the turnaround time of the CBA assay was 3 h, and 6–24 h for ELISA and RIPA.

The single assay adopted per methodology and enrollment of only Chinese patients may limit the generalization of this work. However, our results are consistent with previous studies that had adopted different brand of CBA assays to test MG antibodies in other regions of the world.[13,27] For example, Mirian et al. found that fixed-CBA (EUROIMMUN) had 4% higher sensitivity to AChR antibody for MG compared to RIPA and detected anti-AChR in 21% of SNMG patients with a retrospective diagnostic cohort containing 395 MG and 223 Non-MG in Canada.[13] In our study, we found fixed-CBA (New Terrain) increased 8.2% of AChR antibody positive MG compared to RIPA and detected anti-AChR in 27.5% of RIPA AChR antibody negative MG patients with a cohort containing 2043 MG and 229 Non-MG in China. In addition, we have not compared diagnostic accuracy of AChR or MuSK autoantibody detection by RIPA, ELISA and CBA employing live cell transfections or flow cytometry.[15,27,28] Despite these limitations, our study provides compelling evidencing that

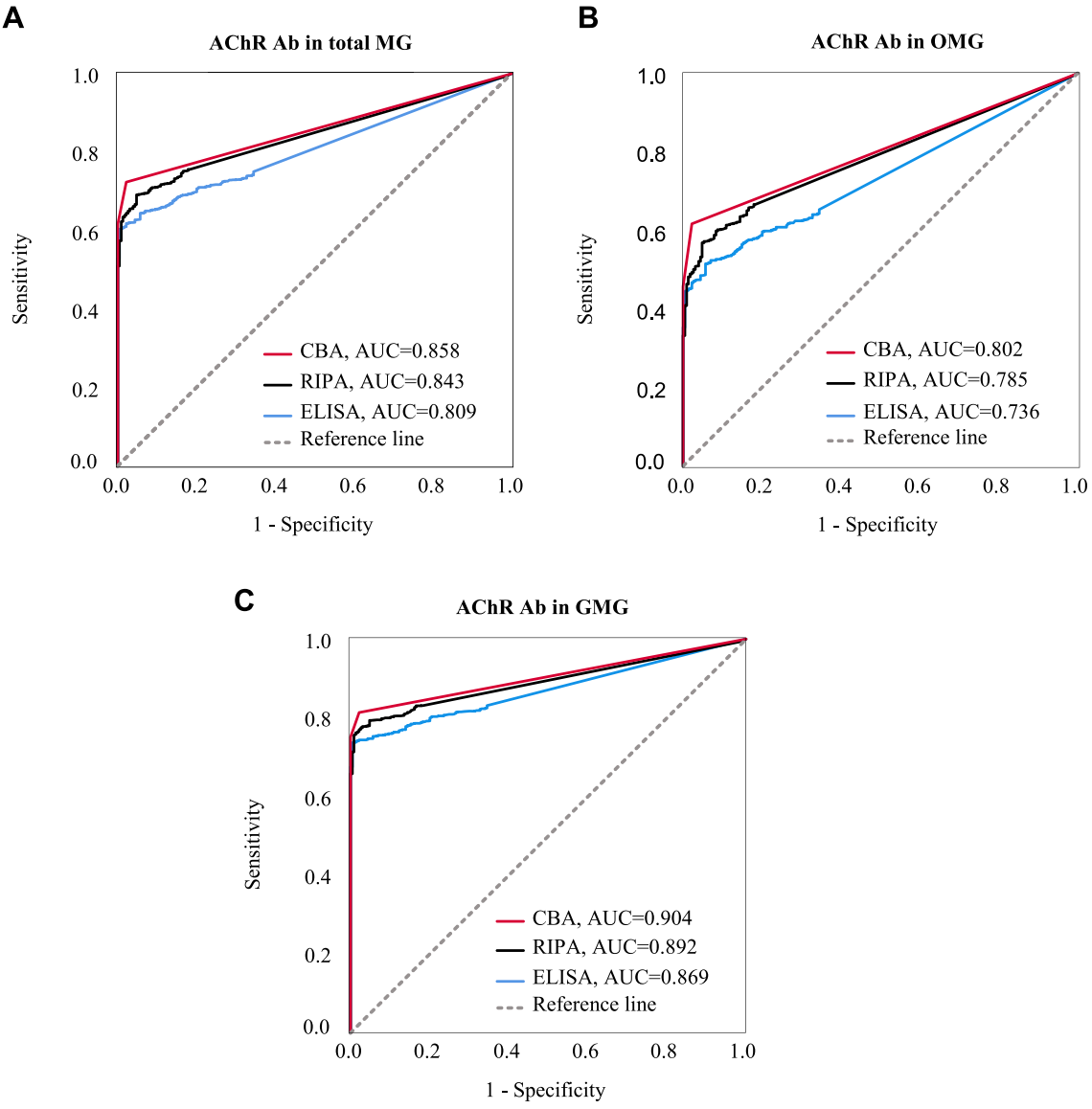

Fig. 2: **The receiver operated characteristic (ROC) curve of AChR antibodies detected by CBA, RIPA, and ELISA.** The row data of AChR antibody tested by CBA (titers), RIPA (nmol/L), and ELISA (nmol/L) were used in the ROC analysis. The area under the ROC curves (AUC, range 0–1) stand for the diagnostic accuracy of CBA, RIPA, and ELISA on AChR antibody. The closer the AUC value is to 1, the better the diagnostic accuracy of the method to be evaluated. The diagnostic accuracy of CBA, RIPA, or ELISA for AChR antibody were analyzed in the total enrolled MG group (**A**), the subset of OMG (**B**) or GMG (**C**). MG, myasthenia gravis; OMG, ocular myasthenia gravis; GMG, generalized myasthenia gravis; RIPA, radioimmunoprecipitation assay; AChR Ab, acetylcholine receptor antibody; ELISA, enzyme-linked immunosorbent assay; CBA, cell-based assay; AUC, area under the curve.

CBA has a higher diagnostic accuracy for the serodiagnosis of AChR and MuSK autoantibodies in MG.

Contributors

F-D.S. and J.P. conceptualized and designed the study; F-D.S. secured funding; F-D.S., Z.L., and C.Z. enforced uniform procedures across the study centres; F-D.S., C.Z., T.C., X.Z., H.Y., F.G., J.F., H.L., S.C., and L.W. recruited patients and acquired clinical data. Z.L., C.Y., and H.L. acquired the investigated test data. F-D.S., J.P., L.Z., Y.P., and C.Z. interpreted the data. F-D.S., L.Z., Y.P., C.Z., and J.P. drafted the manuscript. All authors critically reviewed the manuscript.

Data sharing statement

Supplementary data and study protocol will be available online to others upon publication of this study. The participant data after de-identification will be available between one to three years after the

publication. Requests for data should be directed to the corresponding author and will be assessed for scientific rigour before being approved. A data-sharing agreement is required before data is transferred.

Declaration of interests

All authors declare no competing interests.

Acknowledgment

We thank colleagues from Tianjin Medical University General Hospital and all other research centres for their support of this study; S. Shi and TN. Nguyen for editorial inputs. We also acknowledge Tianjin New Terrain Biotechnology, Inc., Tianjin, China, for providing all CBA kits for autoantibody detection for AChR and MuSK; and logistic supports from New Terrain and KingMed Diagnostics Group Co., Ltd.

Funding/Support: New Terrain Biotechnology, Inc., Tianjin, China.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.lanwpc.2023.100846.

References

1 Gilhus NE. Myasthenia gravis. *N Engl J Med*. 2016;375(26):2570–2581.
2 Vincent A, Palace J, Hilton-Jones D. Myasthenia gravis. *Lancet*. 2001;357(9274):2122–2128.
3 Punga AR, Maddison P, Heckmann JM, Guptill JT, Evoli A. Epidemiology, diagnostics, and biomarkers of autoimmune neuromuscular junction disorders. *Lancet Neurol*. 2022;21(2):176–188.
4 Bril V, Szczudlik A, Vaitkus A, et al. Randomized, double-blind, placebo-controlled trial of the corticosteroid-sparing effects of immunoglobulin in myasthenia gravis. *Neurology*. 2023;100(7):e671–e682.
5 Howard JF Jr, Bril V, Vu T, et al. Safety, efficacy, and tolerability of efgartigimod in patients with generalised myasthenia gravis (ADAPT): a multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet Neurol*. 2021;20(7):526–536.
6 Narayanaswami P, Sanders DB, Wolfe G, et al. International consensus guidance for management of myasthenia gravis: 2020 update. *Neurology*. 2021;96(3):114–122.
7 Gilhus NE, Verschuuren JJ. Myasthenia gravis: subgroup classification and therapeutic strategies. *Lancet Neurol*. 2015;14(10):1023–1036.
8 Sussman J, Farrugia ME, Maddison P, Hill M, Leite MI, Hilton-Jones D. Myasthenia gravis: association of British neurologists' management guidelines. *Pract Neurol*. 2015;15(3):199–206.
9 Leite MI, Jacob S, Viegas S, et al. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. *Brain*. 2008;131(Pt 7):1940–1952.
10 Wolfe GI, Kaminski HJ, Aban IB, et al. Long-term effect of thymectomy plus prednisone versus prednisone alone in patients with non-thymomatous myasthenia gravis: 2-year extension of the MGTX randomised trial. *Lancet Neurol*. 2019;18(3):259–268.
11 Rodriguez Cruz PM, Al-Hajjar M, Huda S, et al. Clinical features and diagnostic usefulness of antibodies to clustered acetylcholine receptors in the diagnosis of seronegative myasthenia gravis. *JAMA Neurol*. 2015;72(6):642–649.
12 Chang T, Leite MI, Senanayake S, et al. Clinical and serological study of myasthenia gravis using both radioimmunoprecipitation and cell-based assays in a South Asian population. *J Neurol Sci*. 2014;343(1–2):82–87.
13 Mirian A, Nicolle MW, Edmond P, Budhram A. Comparison of fixed cell-based assay to radioimmunoprecipitation assay for acetylcholine receptor antibody detection in myasthenia gravis. *J Neurol Sci*. 2022;432:120084.
14 Tsonis AI, Zisimopoulou P, Lazaridis K, et al. MuSK autoantibodies in myasthenia gravis detected by cell based assay–a multinational study. *J Neuroimmunol*. 2015;284:10–17.
15 Damato V, Spagni G, Monte G, et al. Clinical value of cell-based assays in the characterisation of seronegative myasthenia gravis. *J Neurol Neurosurg Psychiatry*. 2022;93(9):995–1000.
16 Masi G, Li Y, Karatz T, et al. The clinical need for clustered AChR cell-based assay testing of seronegative MG. *J Neuroimmunol*. 2022;367:577850.
17 Hewer R, Matthews I, Chen S, et al. A sensitive non-isotopic assay for acetylcholine receptor autoantibodies. *Clin Chim Acta*. 2006;364(1–2):159–166.
18 Martino G, Twaddle G, Brambilla E, Grimaldi LM. Detection of anti-acetylcholine receptor antibody by an ELISA using human receptor from a rhabdomyosarcoma cell line. *Acta Neurol Scand*. 1994;89(1):18–22.
19 Trakas N, Tzartos SJ. Immunostick ELISA for rapid and easy diagnosis of myasthenia gravis. *J Immunol Methods*. 2018;460:107–112.
20 Evoli A, Antonini G, Antozzi C, et al. Italian recommendations for the diagnosis and treatment of myasthenia gravis. *Neurol Sci*. 2019;40(6):1111–1124.
21 Melzer N, Ruck T, Fuhr P, et al. Clinical features, pathogenesis, and treatment of myasthenia gravis: a supplement to the guidelines of the German Neurological Society. *J Neurol*. 2016;263(8):1473–1494.
22 Li Z. China guidelines for the diagnosis and treatment of myasthenia gravis. *Neuroimmunol Neuroinflammation*. 2016;3:1–9.
23 Zhang C, Zhang M, Qiu W, et al. Safety and efficacy of tocilizumab versus azathioprine in highly relapsing neuromyelitis optica spectrum disorder (TANGO): an open-label, multicentre, randomised, phase 2 trial. *Lancet Neurol*. 2020;19(5):391–401.
24 Snellman A, Lantero-Rodriguez J, Emersic A, et al. N-terminal and mid-region tau fragments as fluid biomarkers in neurological diseases. *Brain*. 2022;145(8):2834–2848.
25 Sun F, Tavella-Burka S, Li J, Li Y. Positive acetylcholine receptor antibody in nonmyasthenic patients. *Muscle Nerve*. 2022;65(5):508–512.
26 Leite MI, Coutinho E, Lana-Peixoto M, et al. Myasthenia gravis and neuromyelitis optica spectrum disorder: a multicenter study of 16 patients. *Neurology*. 2012;78(20):1601–1607.
27 Gastaldi M, Scaranzin S, Businaro P, et al. Improving laboratory diagnostics in myasthenia gravis. *Expert Rev Mol Diagn*. 2021;21(6):579–590.
28 Cromar A, Lozier BK, Haven TR, Hill HR. Detection of acetylcholine receptor blocking antibodies by flow cytometry. *Am J Clin Pathol*. 2016;145(1):81–85.