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Original research

Clinical value of cell-based assays in the characterisation of seronegative myasthenia gravis

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ABSTRACT

Objective Patients with myasthenia gravis without acetylcholine receptor (AChR) or muscle-specific kinase (MuSK) antibodies detected by radioimmunoprecipitation assays (RIAs) are classified as seronegative myasthenia gravis (SNMG). Live cell-based assays (l-CBAs) can detect additional antibodies to clustered AChR, MuSK and low-density lipoprotein receptor-related protein 4 (LRP4), but positivity rates are variable and both clinical relevance and utility of CBA platforms remain unclear.

Methods Sera from 82 patients with SNMG were tested by l-CBAs. Human embryonic kidney cells were transfected to individually express clustered AChR, MuSK or LRP4; or transfected to jointly express both clustered adult AChR and MuSK. Sera from 30 and 20 patients positive by RIA for AChR or MuSK antibodies were used as comparators.

Results 53 of 82 (72%) patients with SNMG had generalised and 29 (28%) had ocular disease. The clustered AChR CBA detected antibodies in 16 of 82 patients (19.5%; including 4 patients with solely fetal AChR antibodies), while 7 of 82 (8.5%) patients had MuSK antibodies. A novel exploratory combined adult AChR-MuSK l-CBA efficiently detected all these antibodies in a subset of the SNMG cohort. No LRP4 antibodies were identified. Overall, patients with SNMG with clustered AChR antibodies, CBA-positive MuSK-MG or triple seronegative were younger, had less severe disease than patients with RIA-positive MG and had a better clinical outcome when immunotherapy was started soon after disease onset, although the time interval from onset to immunotherapy was not different when compared with patients with RIA-positive MG.

Conclusion Around one-third of patients with SNMG had AChR or MuSK antibodies by l-CBAs, which were efficiently detected with a combined l-CBA. The results in this large and unselected cohort of patients with MG demonstrate the diagnostic usefulness of performing CBAs and the importance of making these tests more widely available.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ In a subset of patients with seronegative myasthenia gravis (SNMG), acetylcholine receptor (AChR) and muscle-specific kinase (MuSK) autoantibodies are detectable by highly sensitive live cell-based assays (CBAs), but the clinical usefulness of these assays is still unclear.

WHAT THIS STUDY ADDS

⇒ Live CBAs allowed the serological confirmation of both AChR-MG and MuSK-MG in one-third of a large SNMG cohort, which can facilitate treatment decisions. The use of a AChR/MuSK combined assay can be conveniently used in the screening of patients with suspected MG.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our findings validate the addition of CBAs to the diagnostic work-up of MG as they can speed up the serological confirmation in radioimmunoprecipitation assay-negative patients, prompting treatment decisions and improving outcome. These findings could support the definition of specific guidelines for the diagnosis of SNMG including the use of the combined CBAs in the screening of these patients.

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INTRODUCTION

Myasthenia gravis (MG) is the most common disorder of the neuromuscular junction (NMJ), caused by autoantibodies that impair neuromuscular transmission and cause fatigable weakness of voluntary muscles. MG antibodies are directed against different proteins of the NMJ and are associated with distinct disease subtypes.[1] The standard acetylcholine receptor (AChR) radioimmunoprecipitation assay (RIA) can detect antibodies in about 80%–85% of patients with generalised MG and in up to 65% with ocular MG (OMG),[2] with a specificity of 97%–99%. The search for other antibodies in AChR-negative MG led to the identification of muscle-specific kinase (MuSK) antibodies in up to 40% of AChR-negative patients,[3 4] currently detected by RIA in most laboratories. More recently, antibodies against the low-density lipoprotein receptor-related protein 4 (LRP4) have been described in a variable proportion of patients with MG using different assays[5–7] and with low specificity.[8] In the last decade, the development of a high-sensitive live cell-based assay (l-CBA), with human embryonic kidney (HEK) 293 T cells engineered to express specific antigens, led to the detection of 'clustered AChR antibodies'.[9 10] This has improved the diagnostic yield in patients with apparently seronegative MG (SNMG), especially in paediatric

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and ocular cases.[11] However, to date, published studies do not quantify the extent to which l-CBAs contribute to SNMG diagnosis, nor do they ask whether there are novel assay approaches to capture many antigen specificities with a single test. Previous reports were typically based on relatively small SNMG cohorts, focused on the detection of only clustered AChR antibodies,[12–14] and only addressed either solely generalised or ocular symptoms.

Therefore, we studied a large cohort of consecutive patients with MG studied over 23 years at a single tertiary centre in order to assess the presence and clinical significance of antibodies to clustered AChR (fetal or adult form), MuSK and LRP4 by l-CBAs and developed a novel CBA approach.

METHODS

Study population

Our population included 92 patients with MG seen in our clinic between 1997 and 2020 who were negative for AChR and MuSK antibodies by RIA and with an available stored serum sample. The diagnosis of SNMG was based on typical history and signs of MG and by the evidence of neuromuscular transmission impairment on electromyography (EMG) testing (>10% decrement of the compound muscle action potential at repetitive nerve stimulation or increased jitter at single-fibre EMG). All patients with symptoms and signs limited to extrinsic ocular muscles were included if brain MRI was unremarkable. Of the 92 patients first enrolled, 10 were excluded during the follow-up based on: atypical symptoms, which eventually led to an alternative diagnosis (n=4); lost to follow-up in the first 2 years after disease onset (n=4); borderline positive AChR antibodies at retesting by RIA (n=2) (online supplemental material 1). Patients were defined as 'early onset' and 'late onset' based on the cut-off of 50 years of age. Patients <18 years old were considered as 'paediatric'. Patients were considered as having OMG if they sustained solely ocular signs after at least 2 years of follow-up. The severity of MG was evaluated at time of recruitment according to the Myasthenia Gravis Foundation of America clinical classification, while the post-intervention status (PIS) classification[15] was used to assess the clinical state at last follow-up after the recorded treatment.

Antibody detection

The earliest serum sample available for each patient with SNMG, along with 50 healthy controls and 60 disease controls (aquaporin 4 positive-neuromyelitis optica, 15; multiple sclerosis, 10; immune-mediated peripheral neuropathies, 15; amyotrophic lateral sclerosis (ALS), 13; myopathies, 7), were studied by l-CBAs. HEK293 T cells were transfected to express clustered AChR (adult or fetal isoforms), MuSK or LRP4, as previously described.[9 10] A goat anti-human Fc(γ) antibody (Thermo Fisher Scientific, Rockford, Illinois, USA; diluted 1:750) followed by an Alexa Flour (AF) 568 rabbit anti-goat IgG (H+L) antibody (Life Technologies, Eugene, Oregon, USA, diluted 1:750) were used in the assays. Rabbit LRP4 antibodies and AF568-conjugated anti-rabbit IgG were used for testing LRP4 expression and for the evaluation of the LRP4 assay. In developing a combined l-CBA, after optimisation of co-transfection concentration of cDNA for adult AChR, enhanced green fluorescent protein (EGFP)-rapsyn and full-length EGFP-MuSK, live cells were incubated with sera from 30 RIA-positive AChR patients, 20 RIA-positive MuSK patients, 63 patients with SNMG from the study cohort, 50 healthy controls and 60 disease controls. Binding of the fluorescein-labelled secondary antibodies was scored by two blinded observers as previously described[9]: (0)=no labelling; (1)=weak labelling of some transfected cells; (2)=moderate labelling of approximately 20%–50% of transfected cells; (3)=moderate/strong labelling of approximately 50%–80% of transfected cells; (4)=strong labelling of almost all transfected cells.

Statistical analysis

Statistical comparisons of demographics, clinical characteristics and antibody results were performed by Mann-Whitney U test (continuous variables) or Fisher's exact test (categorical variables), as appropriate. Data distribution was assessed using Shapiro-Wilk and Kolmogorov-Smirnov normality tests. Demographic and clinical characteristics of the SNMG population were compared with our RIA-positive MuSK-MG cohort (n=103) and with a sample (n=200) of our cohort of RIA-positive AChR-MG (N=1104), which was extracted based on the decade of disease onset matched with the SNMG population. The number of RIA-positive samples was too small to assess the assay sensitivity of the combined CBA, but specificity was calculated as the proportion (with 95% CI) of control samples (n=110) that resulted negative by the combined CBA. A p value of less than 0.05 was considered statistically significant. Statistical analysis was performed with Graph Pad Prism V.9.1 (Graph Pad Software, La Jolla, California, USA).

RESULTS

Clinical features and antibody profile of the SNMG cohort

Eighty-two patients were included in the final analysis. Antibodies against clustered AChR were found in 16 of 82 (19.5%), including 9 patients with antibodies only binding the adult isoform of AChR and 4 patients with antibodies only binding the fetal isoform. MuSK antibodies were detected in 7 of 82 (8.5%) patients, while no patient in our cohort showed positivity for LRP4 antibodies and no patient was double positive (figure 1A,B, online supplemental material 1). Thus, 59 of 82 (72%) patients with SNMG were seronegative for clustered AChR, MuSK and LRP4 antibodies by l-CBA, and termed triple seronegative (tSNMG). Although only 21 of 82 (26%) patients were sampled at disease onset, 50% (42 of 82) never received any treatment at time of sampling, reducing a possible influence of immunotherapy on the l-CBA results. Moreover, the proportion of patients who were immunotherapy naïve at sampling did not differ between patients positive at l-CBA and tSNMG (Fisher's test, p=0.34).

In total, 51 patients (62%) were female, with median age at disease onset of 38.5 years (range: 6–80 years) and median follow-up of 11.5 years (range: 2–40 years). Patients' age at onset and maximum clinical severity are summarised in figure 1C. Thymectomy was performed in 18 of 82 (22%) patients before sampling, including 8 of 18 patients with thymic hyperplasia and one case with thymic carcinoma. The majority of patients (84%) responded well to long-term cholinesterase inhibitors (ChE-I) treatment and 32% did not receive immunosuppressive therapy during the follow-up. Among the 29 (35%) patients with OMG, 10 (34%) were positive by l-CBA, including 3 patients with MuSK and 3 with solely fetal AChR antibodies (figure 1D).

Clinical characteristics of the serological subgroups

The clinical features and outcome of the serological subgroups are summarised in figure 2.

Patients with clustered AChR antibodies

The majority of patients were early onset (81%), including three with paediatric onset. Thymectomy was performed in three

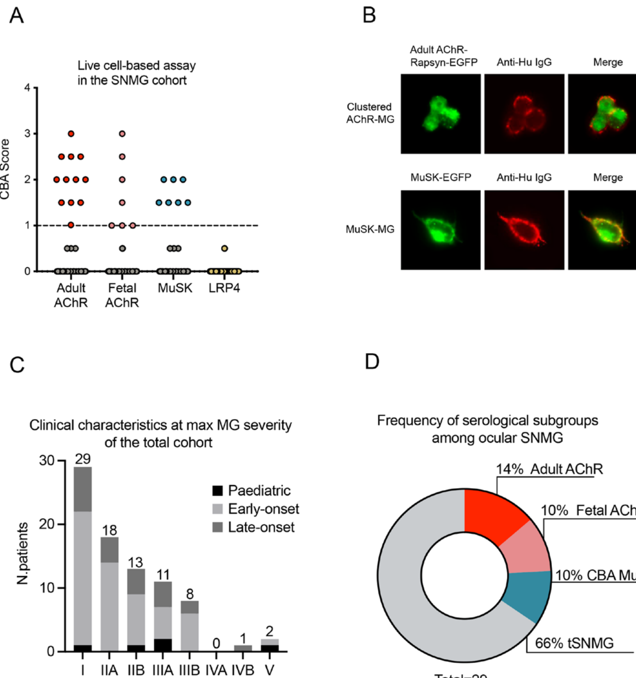

Figure 1 Clinical features and cell-based assay (CBA) results of the SNMG cohort. Live CBA results were based on the degree of cell surface fluorescence and co-localisation of the red fluorescent–labelled secondary antibodies with EGFP-labelled AChRs or MuSK as previously described.[9] Given that LRP4 cDNAs did not contain EGFP, only the cell surface staining was scored (A). Clustered AChR and MuSK IgGs were detected in serum by binding (red) to the surface of live AChR-rapsyn-EGFP or MuSK-EGFP transfected HEK cells (green) (100×) (B). Patients' stratification based on age of onset and maximum disease severity using the MGFA scale (C). Serological characteristics of patients with ocular MG (OMG) in our cohort (D). Patients were defined as 'early onset' and 'late onset' based on the cut-off of 50 years of age. Patients <18 years old were considered as 'paediatric'. Patients were considered as OMG after at least 2 years of follow-up. AChR, acetylcholine receptor; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; LRP4, low-density lipoprotein receptor-related protein 4; MG, myasthenia gravis; MGFA, Myasthenia Gravis Foundation of America; MuSK, muscle-specific kinase; SNMG, seronegative myasthenia gravis; tSNMG, triple seronegative myasthenia gravis.

patients before sampling, with evidence of thymic hyperplasia. All patients responded well to ChE-I, and 7 of 16 (44%) never received immunosuppression. Most of the patients had a mild disease course with a good outcome (eg, PIS minimal manifestations (MMs) or better). In particular, patients with antibodies directed solely to the fetal AChR isoform mostly had OMG with excellent clinical outcomes (** in the heatmap, figure 2). An exception was the case of one patient with paediatric onset, who was negative at an extensive screening for congenital myasthenic syndromes (CMS), refused immunotherapy and developed fixed ophthalmoparesis in spite of a partial response to 3,4-diaminopyridine.

Patients with MuSK antibodies

All patients had a mild disease course, and typical bulbar weakness at peak disease severity was found in three out of seven patients. Interestingly, three patients had isolated ocular disturbance characterised by bilateral ophthalmoparesis. Most patients (six out of seven) had a good outcome after a short course of steroids, and two out of seven patients responded well to ChE-I, while five out of seven patients did not show any cholinergic hypersensitivity symptoms. An exception was the case of a young woman who suffered from predominant limb weakness, refused adequate steroid treatment and had significant benefit after a trial of salbutamol. Patients with MuSK antibodies detectable only by CBA were compared with our RIA-positive MuSK-MG cohort (n=103). Although demographics, treatment response and follow-up were similar between these two subgroups, patients with only CBA-positive MuSK antibodies had milder disease course (online supplemental material 2), also because of the presence of purely OMG cases, otherwise absent in our RIA-positive MuSK-MG population.

Patients with tSNMG

Fifty-nine patients were seronegative for clustered AChR, MuSK and LRP4 antibodies. Thirty-nine (65%) patients were female, with age of onset ranging between 6 and 72 years (median 40 years) and mild disease severity. Thymectomy was performed in 12 patients, with 5 of 12 (42%) having thymic hyperplasia and 1 with thymic carcinoma. Thirty-nine of fifty-nine (66%) patients were in MM or better at last follow-up.

Clinical outcome analysis

We analysed the factors that could be associated with good clinical outcome (eg, PIS MM or better at last follow-up) in our SNMG cohort and we found that patients who received early immunotherapy achieved a better outcome than those in whom immunotherapy was delayed ($p=0.039$, univariable analysis, table 1). We then compared patients who had clustered AChR antibodies or tSNMG with a sample of 200 RIA-positive AChR patients attending our centre, and we found that the former groups were younger, more frequently ocular and with a milder disease course than RIA-positive patients, despite resulting in a similar outcome (online supplemental material 3 and 4). We asked whether this discrepancy could be explained by diagnostic hesitancy resulting in delayed or lack of immunotherapy in the SNMG cohort. We found that while the rate of immunotherapy among AChR RIA-positive patients was higher than in the SNMG cohort ($p<0.001$, Mann-Whitney rank test), the time interval from onset to immunotherapy was not different between these subgroups.

AChR/MuSK combined l-CBA

We developed an exploratory combined assay which, simultaneously, exposes patients' antibodies to both adult AChR and MuSK expressing HEK293 T cells. By testing RIA-positive AChR and MuSK samples along with healthy controls, we showed that this CBA could detect AChR and MuSK antibodies in all RIA-positive samples and retained an excellent specificity (figure 3A,B). Indeed, specificity was 100% (one-sided 95% CI=97.3% to 100%) for both adult AChR and MuSK CBA. However, testing of larger cohort of healthy and disease controls is needed to confirm these data. Next, we applied the combined assay to a proportion of patients (n=63) from our SNMG cohort (which did not include the fetal AChR antibody-positive patients), finding the same sensitivity to the single assay but no further positive cases among the patients with tSNMG (figure 3C,D).

DISCUSSION

Despite advances in the detection of antibodies in MG, around 10% of patients are still negative on RIA for AChR or MuSK

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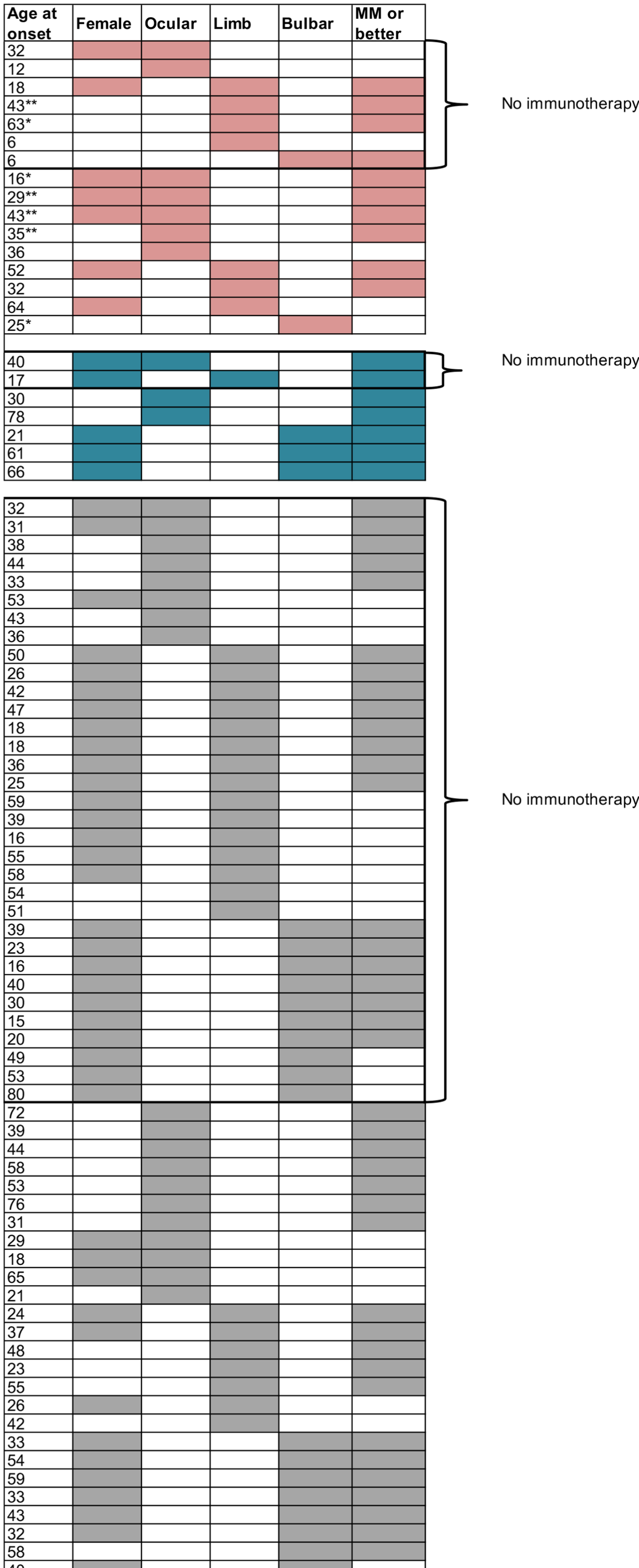

Figure 2 Clinical characteristics of the serological MG subgroups. Heatmap of clinical features and outcome of individual cases from our SNMG cohort stratified by antibody status and need for immunotherapy. *Positivity for both adult and fetal AChR isoform. **Positivity for only fetal AChR. 'Ocular', 'bulbar' and 'limb' were the muscle groups predominantly affected at maximum disease severity. AChR, acetylcholine receptor; MG, myasthenia gravis; MM, minimal manifestation; MuSK, muscle-specific kinase; SNMG, seronegative myasthenia gravis; tSNMG, triple seronegative myasthenia gravis.

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Table 1 Comparison between patients achieving a good clinical outcome (MM or better) and those still symptomatic at last follow-up

SNMG cohort (n=82)	MM or better (n=56)	Symptomatic (n=26)	P value
Median age at onset (IQR), years	36.5 (25.5–49)	41 (26–54)	0.4368
Female	35 (63%)	16 (62%)	1.000
Median follow-up (IQR), months	12 (6–18)	9.5 (4–19)	0.7189
Clustered AChR+	10 (18%)	6 (23%)	0.565
MuSK-CBA+	7 (12.5%)	0	0.091
tSNMG	39 (70%)	20 (77%)	0.495
Max disease severity			
Ocular	19 (34%)	10 (39%)	0.8048
Mild	24 (43%)	7 (27%)	0.2228
Moderate	10 (18%)	9 (35%)	0.1579
Severe	1 (2%)	0	1.000
Crisis	2 (4%)	0	1.000
Bulbar	19 (34%)	5 (19%)	0.173
Immunotherapy			
None	19 (34%)	12 (46%)	0.3328
CS	25 (45%)	10 (39%)	0.6389
CS+IS	12 (21%)	4 (15%)	0.7653
Median time from onset to therapy (IQR), months	6.5 (1–14)	34 (12–89)	0.0392

AChR, acetylcholine receptor; CBA, cell-based assay; CS, corticosteroids; IS, immunosuppressants; MM, minimal manifestation; MuSK, muscle-specific kinase; SNMG, seronegative myasthenia gravis; tSNMG, triple seronegative myasthenia gravis.

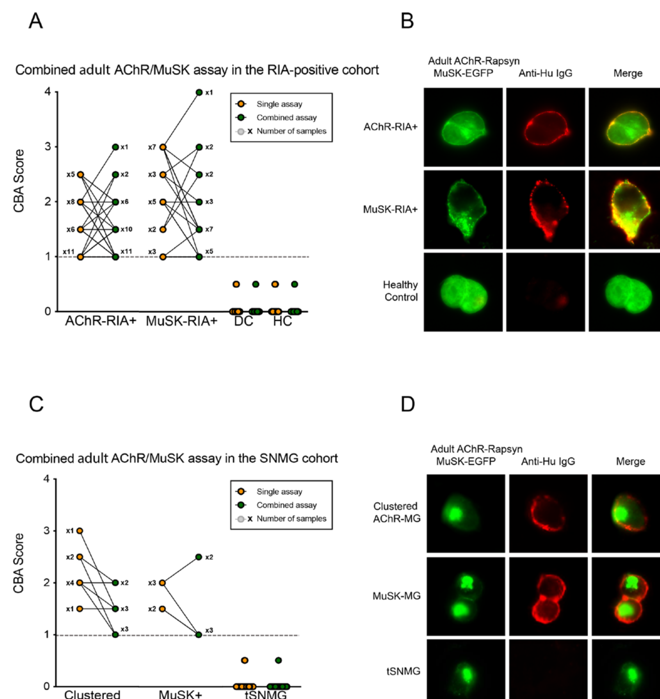

Figure 3 Single and combined adult AChR/MuSK assay results. Comparison of the l-CBA score of the single assay versus the combined assay in the RIA-positive cohort (n=30 AChR-RIA+, n=20 MuSK-RIA+ samples) (A) and in the SNMG cohort (n=63 samples) (C) compared with DC (n=60) and HC (n=50). The combined CBA retained the same specificity of the single assay. Anti-human Fc(γ) antibody binding (red staining) of AChR-MG, MuSK-MG and HC sera to AChR-EGFP-rapsyn+MuSK EGFP (green) expressed on HEK cells. Merge of anti-human Fc(γ) IgG and AChR+MuSK EGFP images demonstrates co-localisation of MuSK-MG or AChR-MG sera but not HC sera (100×) (B,D). AChR, acetylcholine receptor; DC, disease controls; EGFP, enhanced green fluorescent protein; HC, healthy controls; HEK, human embryonic kidney; l-CBA, live cell-based assay; MG, myasthenia gravis; MuSK, muscle-specific kinase; RIA, radioimmunoprecipitation assay; SNMG, seronegative myasthenia gravis; tSNMG, triple seronegative myasthenia gravis.

antibodies. Here, we found that one-third of RIA-negative patients, with either generalised or ocular MG, were positive for clustered adult/fetal AChR or MuSK antibodies. Moreover, the use of l-CBA was successful in discriminating the antibody specificity for fetal or adult AChR, with nine patients harbouring antibodies only against the adult isoform of AChR and four patients (mostly with OMG) who only had antibodies against fetal AChR. None of the patients' newborns had a history of neonatal MG. Indeed, MG antibodies binding preferentially to the fetal or the adult AChR subtype are rare but may be relevant to the clinical phenotype.[16–18] Whether this is in keeping with the known expression of fetal AChRs in extraocular muscles[19] merits further study.

The lack of LRP4 antibodies in our population was not altogether unexpected. The incidence of LRP4 antibodies in SNMG studies from different countries is generally low and variable.[20–22] Moreover, the variable incidence reported among patients with ALS (23% in Greek/Italian patients, 10% in US patients, 5% in Chinese patients) could reflect the need for improvements in assay specificities.[8 21 22] It is notable that here no LRP4 antibodies were detected in any disease controls including 15 patients with ALS, as also reported in a previous Italian multicentre study, in which 14% of patients with MG were found to have LRP4 antibodies.[6]

The results in this large cohort of patients with SNMG support the clinical value of performing CBAs in MG and the importance of making these tests more widely available. On one hand, l-CBAs represent a useful diagnostic tool to confirm MG diagnosis in SNMG and could favour early immunotherapy likely resulting in clinical outcome improvement. On the other hand, the use of a more sensitive antibody assay could allow the confirmation of a positive serostatus, making these patients eligible for clinical trials and more targeted and effective treatments, including therapeutic thymectomy. Moreover, despite CBAs being currently limited to selected laboratories,[23] they do not require radioactivity and could be easily employed at larger scale thanks to the recent commercialisation of fixed CBAs (f-CBAs) for adult/fetal AChR and MuSK. F-CBA has the advantage of not requiring cell culture facilities and transfection steps and proved reliable in clinically defined patient groups.[24] However, more studies are needed to test their sensitivity in detecting low affinity antibodies, as in the case of clustered AChR antibodies.[25]

In addition, in the present study, we designed an exploratory combined clustered adult AChR/MuSK l-CBA, which retained the same specificity of the individual assays. The current study was not aimed to assess the combined CBA metrics in comparison with RIA and therefore was not powered for such purpose and not designed to screen large and unselected cohort of samples. However, if applied to a larger sample size, we anticipate this assay could be used as an efficient diagnostic tool in patients with SNMG.

Given the reservations regarding, in particular, LRP4 antibody tests, CBA testing should be reserved for patients with a high index clinical suspect of MG, preferably with neurophysiological or pharmacological evidence of an NMJ defect. Although these findings are encouraging, this study has some

limitations. First, its retrospective design; second, the sampling of half of the patients during immunotherapy; third, the lack of screening for CMS in most of our tSNMG cases.[26] Further studies should focus on recruiting prospective large series of SNMG immunosuppression-naive cohorts and on methodological standardisation of the applied assays. At present, these assays should be reserved for patients with SNMG after the exclusion of alternative diagnoses. Moreover, the diagnostic work-up for CMS has now been improved by the availability of multigene screening panels and should be performed in patients without a clear response to immunotherapy.

In clinical practice, the diagnosis of MG can be challenging in patients with negative results on AChR and MuSK antibody using standard assays, highlighting the need for a consensus on clinical and electrophysiological criteria for the diagnosis of SNMG and the importance of including CBAs in the diagnostic work-up of these patients.

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Contributors Study concept and design—VD and AE. Acquisition of data—VD, GS, GM, MW, LJ, SF, TS, RI and AE. Analysis and interpretation of data—VD, GS, GM, MW, LJ, SF, TS, PW, SRI, AV and AE. Drafting of the manuscript—VD and AE. Critical revision of the manuscript for important intellectual content—VD, GS, PW, SRI, AV and AE. Study supervision—PW, SRI, AV and AE. VD and AE act as guarantors.

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Disclaimer The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Competing interests AV and the University of Oxford held a patent for detection of MuSK antibody assays (expired 2020), licensed to Athena Diagnostics; AV received a proportion of royalties. SRI and PW are co-applicants and receive royalties on patent application WO/2010/046716 entitled 'Neurological Autoimmune Disorders' (the patent has been licensed for the development of assays for LGI1 and other VGKC-complex antibodies) and have filed two other patents regarding autoantibody diagnostic algorithms.

Patient consent for publication Not required.

Ethics approval This study involves human participants and was conducted in conformation with the Helsinki Declaration and was approved by the Ethics Committee of the Università Cattolica del Sacro Cuore and the Nuffield Department of Clinical Neuroscience of Oxford (protocol #26739/13/ID.215, REC16/YH/0013, REC16/SC/0224 and REC14/SC/005). Participants gave informed consent to participate in the study before taking part.

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Data availability statement Data are available upon reasonable request.

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REFERENCES

1. Evoli A, Spagni G, Monte G, *et al*. Heterogeneity in myasthenia gravis: considerations for disease management. *Expert Rev Clin Immunol* 2021;17:761–71.
2. Monte G, Spagni G, Damato V, *et al*. Acetylcholine receptor antibody positivity rate in ocular myasthenia gravis: a matter of age? *J Neurol* 2021;268:1803–7.
3. Hoch W, McConville J, Helms S, *et al*. Auto-Antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. *Nat Med* 2001;7:365–8.
4. Evoli A, Tonali PA, Padua L, *et al*. Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis. *Brain* 2003;126:2304–11.
5. Zisimopoulou P, Evangelakou P, Tzartos J, *et al*. A comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 in myasthenia gravis. *J Autoimmun* 2014;52:139–45.
6. Marino M, Scuderi F, Samengo D, *et al*. Flow cytofluorimetric analysis of Anti-LRP4 (LDL receptor-related protein 4) autoantibodies in Italian patients with myasthenia gravis. *PLoS One* 2015;10:e0135378.
7. Zhang B, Tzartos JS, Belimezi M, *et al*. Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. *Arch Neurol* 2012;69:445–51.
8. Tzartos JS, Zisimopoulou P, Rentzos M, *et al*. LRP 4 antibodies in serum and CSF from amyotrophic lateral sclerosis patients. *Ann Clin Transl Neurol* 2014;1:80–7.
9. Leite MI, Jacob S, Viegas S, *et al*. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis†. *Brain* 2008;131:1940–52.
10. Jacob S, Viegas S, Leite MI, *et al*. Presence and pathogenic relevance of antibodies to clustered acetylcholine receptor in ocular and generalized myasthenia gravis. *Arch Neurol* 2012;69:994–1001.
11. Rodríguez 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;72:642–9.
12. Devic P, Petiot P, Simonet T, *et al*. Antibodies to clustered acetylcholine receptor: expanding the phenotype. *Eur J Neurol* 2014;21:130–4.
13. Zhao G, Wang X, Yu X, *et al*. Clinical application of clustered-AChR for the detection of SNMG. *Sci Rep* 2015;5:10193.
14. Huda S, Woodhall MR, Vincent A, *et al*. Characteristics of acetylcholine-receptor-antibody-negative myasthenia gravis in a South African cohort. *Muscle Nerve* 2016;54:1023–9.
15. Jaretzki A, Barohn RJ, Ernstoff RM. Myasthenia gravis: recommendations for clinical research standards. *Neurology* 2000;55:16–23.
16. Shi Q-G, Wang Z-H, Ma X-W, *et al*. Clinical significance of detection of antibodies to fetal and adult acetylcholine receptors in myasthenia gravis. *Neurosci Bull* 2012;28:469–74.
17. Nagaishi A, Narita T, Woodhall M, *et al*. Autoantibodies in Japanese patients with ocular myasthenia gravis. *Muscle Nerve* 2021;63:262-267.
18. Ohta K, Fujinami A, Saida T, *et al*. Frequency of anti-AChR ϵ subunit-specific antibodies in Mg. *Autoimmunity* 2003;36:151–4.
19. MacLennan C, Beeson D, Buijs A-M, *et al*. Acetylcholine receptor expression in human extraocular muscles and their susceptibility to myasthenia gravis. *Ann Neurol* 1997;41:423–31.
20. Li Y, Zhang Y, Cai G, *et al*. Anti-LRP4 autoantibodies in Chinese patients with myasthenia gravis. *Muscle Nerve* 2017;56:938–42.
21. Rivner MH, Liu S, Quarles B, *et al*. Agrin and low-density lipoprotein-related receptor protein 4 antibodies in amyotrophic lateral sclerosis patients. *Muscle Nerve* 2017;55:430–2.
22. Lei L, Shen X-M, Wang S-Y, *et al*. Presence of antibodies against low-density lipoprotein receptor-related protein 4 and impairment of neuromuscular junction in a Chinese cohort of amyotrophic lateral sclerosis. *Chin Med J* 2019;132:1487–9.
23. Rodriguez Cruz PM, Huda S, López-Ruiz P, *et al*. Use of cell-based assays in myasthenia gravis and other antibody-mediated diseases. *Exp Neurol* 2015;270:66–71.
24. Waters P, Reindl M, Saiz A, *et al*. Multicentre comparison of a diagnostic assay: aquaporin-4 antibodies in neuromyelitis optica. *J Neurol Neurosurg Psychiatry* 2016;87:1005–15.
25. Mirian A, Nicolle MW, Edmond P, *et al*. Comparison of fixed cell-based assay to radioimmunoprecipitation assay for acetylcholine receptor antibody detection in myasthenia gravis. *J Neurol Sci* 2022;432:120084.
26. Kao JC, Milone M, Selcen D, *et al*. Congenital myasthenic syndromes in adult neurology clinic: a long road to diagnosis and therapy. *Neurology* 2018;91:e1770–7.

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