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Clinical features of LRP4/agrin-antibody–positive myasthenia gravis: A multicenter study

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Abstract

Introduction: Our aim in this study was to identify the prevalence and clinical characteristics of LRP4/agrin-antibody–positive double-seronegative myasthenia gravis (DNMG).

Methods: DNMG patients at 16 sites in the United States were tested for LRP4 and agrin antibodies, and the clinical data were collected.

Results: Of 181 DNMG patients, 27 (14.9%) were positive for either low-density lipoprotein receptor–related protein 4 (LRP4) or agrin antibodies. Twenty-three DNMG patients (12.7%) were positive for both antibodies. More antibody-positive patients presented with generalized symptoms (69%) compared with antibody-negative patients (43%) ($P \le .02$). Antibody-positive patients' maximum classification on the Myasthenia Gravis Foundation of America (MGFA) scale was significantly higher than that for antibody-negative patients ($P \le .005$). Seventy percent of antibody-positive patients were classified as MGFA class III, IV, or V compared with 39% of antibody-negative patients. Most LRP4- and agrin-antibody–positive patients (24 of 27, 89%) developed generalized myasthenia gravis (MG), but with standard MG treatment

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81.5% (22 of 27) improved to MGFA class I or II during a mean follow-up of 11 years.

Discussion: Antibody-positive patients had more severe clinical disease than antibody-negative patients. Most DNMG patients responded to standard therapy regardless of antibody status.

KEYWORDS

agrin, clinical features, LRP4, myasthenia gravis, neuromuscular transmission disorders, seronegative myasthenia gravis

1 | INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease in which antibodies directed against the neuromuscular junction cause fatigable weakness.[1] In approximately 80% of patients with generalized MG, antibodies to acetylcholine receptors (AChRs) have been identified.[2] In another 10% of generalized MG patients, antibodies to muscle-specific kinase (MuSK) have been reported.[3] Although a small percentage of generalized MG patients may have genetic defects (congenital myasthenic syndromes [CMSs]), rather than an immune pathogenesis, most of the remaining 10% of double-seronegative MG patients (DNMG) are believed to have an autoimmune etiology, as they respond to immunosuppressant and immunomodulatory therapies.[4]

Agrin is a proteoglycan utilized by motor neurons to instruct neuromuscular junction formation and maintenance.[5] Agrin binds to low-density lipoprotein receptor–related protein 4 (LRP4) and activates MuSK. The ensuing intracellular pathways lead to the aggregation of AChR at the neuromuscular junction.[6–9] Antibodies to LRP4 and agrin have been found in MG patients, particularly DNMG,[10–14] and have been shown to produce MG in experimental animals.[15–17] This makes it likely that anti-agrin and LRP4 antibodies may be pathogenic factors for DNMG. Also, mutations in these proteins have been reported in

CMSs.[18–23] To date, there have been 225 patients described in the literature who have MG associated with LRP4 antibodies,[10–12,24–33] but clinical data were described for only 133 of these patients. Thirty-six agrin-antibody–associated MG cases have been described,[13,14] only 5 of which have clinical data.[13] The data on DNMG patients with LRP4 and/or agrin antibodies are even more limited.

The prevalence rates vary dramatically in the literature. For example, the prevalence of LRP4 antibodies in MG patients was found to vary from 0.45% to 40%, and in DNMG patients from 0.14% to 50%.[10–12,25–30,32] The prevalence of anti-agrin antibodies in MG patients varied from 7.5% to 14.9%, and in DNMG patients fluctuated between 0% and 50%.[13,14,26] Little is known regarding the clinical value of these antibodies. In this study, we assessed prevalence, clinical features, and prognosis in carefully screened DNMG patients in the United States who have antibodies to LRP4 and/or agrin.

2 | METHODS

Patients over the age of 21 seen between May 2016 and June 2018 were enrolled at 16 clinical sites (Figure 1). Eligible patients had clinical signs of MG, either an abnormal repetitive stimulation study and/

FIGURE 1 Sequence of study visits and details on which procedures are performed at each visit. Abbreviations: ACHR, acetylcholine receptor; IVIG, intravenous immunoglobulin; LRP4, low-density lipoprotein receptor–related protein 4; PLEX, plasma exchange; MG, myathenia gravis; MuSK, muscle-specific kinase; QMG, quantitative myasthenia gravis score
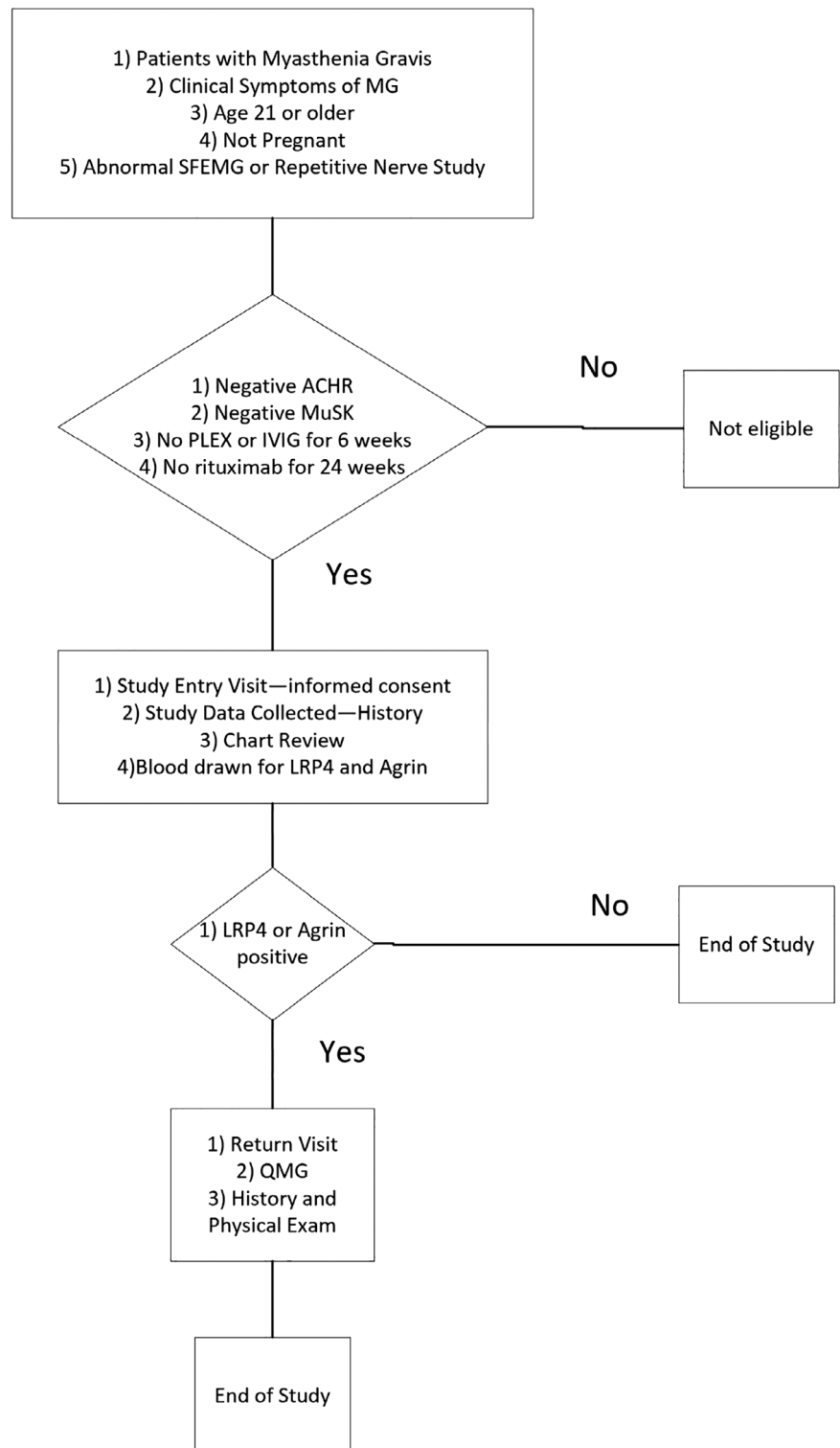
or single-fiber electromyogram, and tested negative for both AChR and MuSK antibodies. All patients provided informed consent for participation in the study. Patients were excluded if they received either plasma exchange or intravenous immunoglobulin (IVIg) within 6 weeks of their antibody test or rituximab within 24 weeks of their test. Pregnant patients were also excluded from study participation. Normal control samples were obtained from 106 random blood donors. The study was approved by the institutional review board at each participating clinical site.

Chart review was performed on patients who met study entry criteria. They were graded according to the Myasthenia Gravis Foundation of America (MGFA) classification during two time periods[34]: (1) on enrollment in the study (study entry); and (2) when their MGFA classification was at its highest (maximum severity). LRP4 and/or

agrin-antibody–positive DNMG patients (LAPMG) were also graded at disease onset. Blood samples were tested for LRP4 and agrin by enzyme-linked immunoassay (ELISA), as described in what follows. Investigators performing the ELISA were blinded to whether samples were from MG patients or controls and the origin of the samples. LAPMG patients underwent an additional detailed history, physical and neurological examinations, and quantitative myasthenia gravis (QMG) testing[35] during a second return examination (return exam). DNMG patients negative for LRP4 and/or agrin antibodies, quadruple-negative MG (QNMG), underwent chart review only.

ELISA assays for agrin and LPR4 antibodies were performed as described elsewhere.[12,14] Briefly, 96-well Nunc MaxiSorp plates (Thermo Fisher Scientific, Waltham, Massachusetts) were incubated with recombinant agrin or LRP4 in carbonate buffer (500 mmol/L, pH 9.6) overnight at 4°C. After rinsing with 0.3% Tween in Tris-buffered saline (TBST) for agrin antibody and 0.1% TBST for LRP4 antibody, wells were incubated in blocking buffer (5% milk in 0.3% TBST) for 2 hours at room temperature. Sera were diluted in 1:100 with the blocking buffer and added to the wells for incubation overnight at 4°C. The wells were washed six times with respective TBST buffers on a shaking platform and then incubated with anti-human immunoglobulin antibodies (1:2000 dilution in 2.5% milk/0.3% TBST) for 1 or 2 hours at room temperature. After washing, the wells were incubated with substrate buffer containing 10 mol/L diethanolamine, 0.5 mmol/L MgCl$_2$, and p-nitrophenylphosphate for 5 to 15 minutes at room temperature. Samples were subjected to optical density readings at 405 nm.

Antibody testing was considered abnormal if the value was 2.5 standard deviations above values for normal control samples. Data were entered by each site on the ONCORE database system at Augusta University. Data were reviewed for completeness and analyzed using Excel (Microsoft Corp, Redmond, Washington). Statistical testing was done in Excel with QI Macro (KnowWare International, Denver, Colorado) and SPSS version 25 (IBM, Armonk, New York). Qualitative statistics were evaluated using a two-tailed Fisher exact test. A two-tailed t test was also used for quantitative data.

3 | RESULTS

Of 181 DNMG patients who were tested for LRP4 and agrin antibodies, 154 (85.1%) were negative for autoantibodies to LRP4 and agrin (QNMG) and 27 (14.9%) were positive for at least one of these two autoantibodies (LAPMG). Twenty-three (12.7%) patients were positive for both LRP4 and agrin antibodies. Three patients were positive for only agrin antibodies, whereas one patient was positive for only LRP4 antibodies. Because only a small number of patients were positive for only a single antibody, we focused on the 27 patients

TABLE 1 Features of MG

	QNMG		LAPMG			
	Number	Percent	Number	Percent	Significance	Percent of group
Clinical features						
Age of onset	46.75 years		44.15 years		$P = .47$ (NS)	
Disease duration	11.4 years		11.3 years		$P = .94$ (NS)	
Female	104	67.53%	16	59.26%	$P = .40$ (NS)	
Ocular onset	88	57.14%	8	30.77%	$P \leq .02^*$	
Ocular study entry	55	35.71%	6	22.22%	$P = .19$ (NS)	
Caucasian	111	72.08%	21	77.78%		18.92%
Black	28	18.18%	4	14.81%	$P = .87$ (NS)	14.29%
Asian	6	3.90%	2	7.41%		33.33%
Hispanic	4	2.60%	0	0.00%		0.00%
Drug treatments						
PLEx	37	24.03%	8	29.63%	$P = .63$ (NS)	
IVIg	36	23.38%	4	14.81%	$P = .45$ (NS)	
Rituximab	18	11.69%	2	7.41%	$P = .74$ (NS)	
Pyridostigmine	139	90.26%	24	88.89%	$P = .74$ (NS)	
Prednisone	105	68.18%	19	70.37%	$P = 1.00$ (NS)	
Azathioprine	33	21.43%	5	18.52%	$P = 1.00$ (NS)	
Mycophenolate mofetil	49	31.82%	12	44.44%	$P = .27$ (NS)	
Methotrexate	20	12.99%	3	11.11%	$P = 1.00$ (NS)	

Abbreviations: IVIg, intravenous immunoglobulin; LAPMG, double-seronegative myasthenia gravis with positivity for low-density lipoprotein receptor–related protein 4 and/or agrin antibodies; MG, myathenia gravis; NS, not statistically significant; PLEx, plasma exchange; QNMG, quadruple seronegative myasthenia gravis.
*Statistically significant ($P < .05$).

who were positive for either LRP4 or agrin antibodies (LAPMG) and compared their results with those negative for these antibodies (QNMG).

Table 1 summarizes the basic data of our DNMG population. The majority of our patients were female. There was no significant difference in gender between the LAPMG and QNMG groups, even though there was a slightly higher percentage of females in the QNMG group. Caucasian/white patients made up the bulk of our population, but other ethnic groups were also represented. There was no significant difference in ethnicity between the two groups. The mean age at disease onset was 46.3 years. This did not differ significantly between the LAPMG and QNMG groups. The mean time between disease onset and entry into the study was 11.4 years. This latency did not differ significantly between the LAPMG and QNMG groups. The first, second, and third quartiles for disease duration in the LAPMG group were 3.3, 8.5, and 15.7 years, respectively, and for the QNMG group 3.9, 7.8, and 14.4 years. Most patients had an ocular-only onset. Significantly fewer LAPMG patients had an ocular onset compared with the QNMG group. Therefore, LAPMG patients are more likely to have generalized MG at disease onset. By the time patients entered the study (study entry),
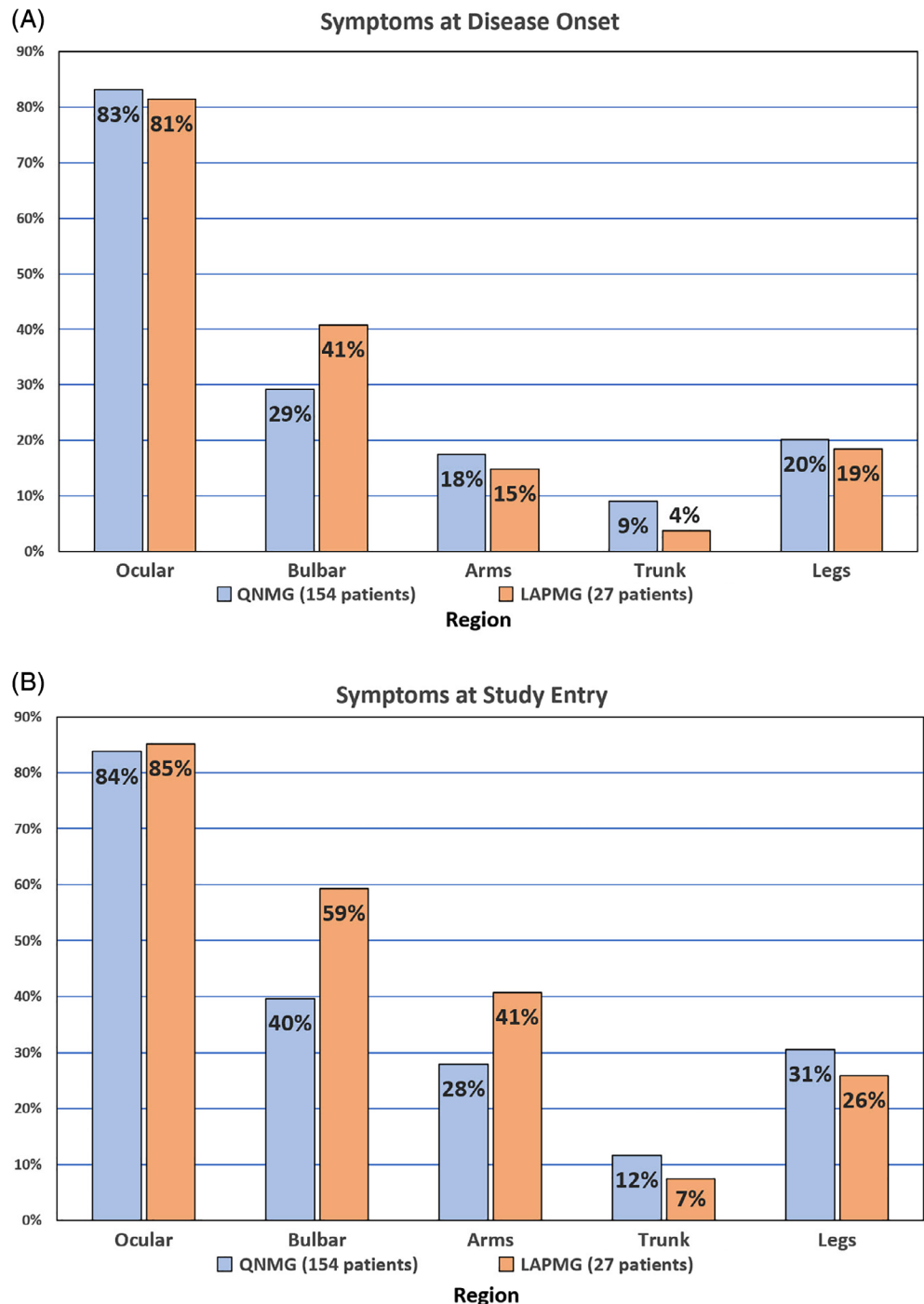

FIGURE 2 Symptoms of both antibody-positive and -negative patients at two time periods. A, Disease onset. B, Time of study visit (study entry exam). Abbreviations: DNMG, double-seronegative myasthenia gravis (negative for AChR and MuSK); LAPMG, double-seronegative myasthenia gravis with positivity for LRP4 and/or agrin antibodies [Color figure can be viewed at wileyonlinelibrary.com]

there was no significant difference in the percentage of patients with ocular-only symptoms between the two groups (Table 1).

The symptoms at disease onset and during study entry are detailed on Figure 2. Symptoms did not differ significantly between the two groups. LAPMG patients tend to have more bulbar symptoms, but this difference was neither significant at disease onset nor at the time of study entry. At the study entry visit, LAPMG patients tended to have more upper extremity symptoms but this difference was not significant ($P = .18$, not statistically significant [NS]).
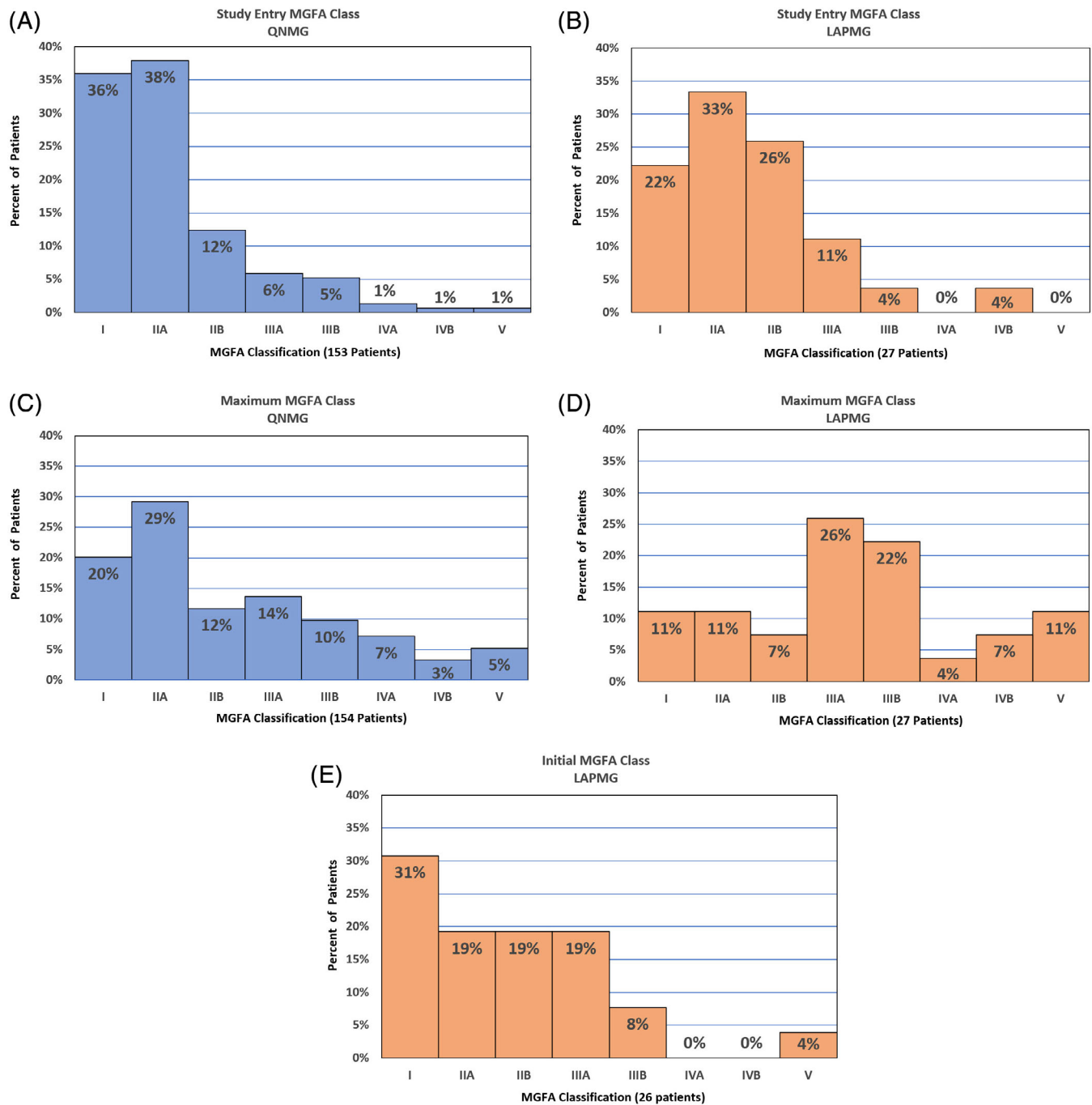

FIGURE 3 Percentage of patients in each MGFA class for three time periods. A, Study entry MGFA class for antibody-negative patients. B, Study entry MGFA class for antibody-positive patients. C, Maximal MGFA class for antibody-negative patients. D, Maximal MGFA class for antibody-positive patients. E, MGFA class at disease onset for antibody-positive patients. Abbreviations: LAPMG, double-seronegative myasthenia gravis with positivity for LRP4 and/or agrin antibodies; MGFA, Myasthenia Gravis Foundation of America; QNMG, quadruple seronegative myasthenia gravis (negative for acetylcholine receptor, muscle-specific kinase, low-density lipoprotein receptor–related protein 4, and agrin) [Color figure can be viewed at wileyonlinelibrary.com]

The MGFA classification of our patients at study entry did not differ between the two groups (Figure 3A,B). Most patients (85.6%) in our total MG population had either ocular or mild, generalized symptoms. Most (81.5%) in the LAPMG group had either ocular or mild disease at the time of study entry. MGFA classification at disease onset was studied only in the LAPMG group (Figure 3E). The majority (69.2%) of LAPMG patients were MGFA class I or II, whereas the others (30.8%) were MGFA class III, IV, or V. One patient presented as MGFA class V.

At the time of maximum disease severity (Figure 3C,D), 56.4% of all DNMG patients had ocular or mild disease severity (MGFA class I or II). In the LAPMG group, 70.4% of subjects were MGFA class III or higher as compared with 39% in the QNMG group (Figure 4). This difference was highly significant ($P = .003$) during this time period, but not at the time of study entry (Figure 4).

MGFA classification at the time of study entry was similar for the LAPMG and QNMG groups (Figure 3A,B) ($P = .26$, NS). Even when combining a (axial/limb muscle predominance) and b (bulbar muscle predominance) at each classification level, the results were not significant ($P = .45$, NS). At the time of maximal disease severity (Figure 3C, D), LAPMG patients had more severe disease than QNMG patients. This difference was not significant ($P = .0608$) when each classification was considered by itself, but if the patients are grouped by severity (combining the a and b groups at each severity level), then the LAPMG group would be significantly worse than the QNMG group ($P = .0253$). Three patients (11.1%) in the LAPMG group needed mechanical ventilation, which was double the percentage (5.2%) requiring ventilation in the QNMG group, but this difference was not significant ($P = .21$). There was no significant difference in the number of patients classified as a (axial/limb muscle predominance) vs b (bulbar muscle predominance) between the two groups ($P = .22$).

The patients were treated with standard therapies for MG, regardless of antibody status (Table 1). There was no significant difference in the medications used by the two groups. Most patients (90.1%) were treated with pyridostigmine. Plasma exchange was used in 24.9%, IVIg in 22.1%, and rituximab in 11.0%. Prednisone was used in 68.5%, azathioprine in 21.0%, mycophenolate mofetil in 33.7%, and methotrexate in 12.7% of patients. The majority (65.4%) of LAPMG patients responded to pyridostigmine, 23.1% worsened, and the remainder (11.5%) had unclear responses. Based on improvement in the MGFA grade between the time of maximum disease severity and study entry (Figure 3), it appears that most LAPMG patients ($P < .001$) as well as most QNMG patients ($P < .001$) responded to standard treatments for MG. Although disease fluctuation may account for some of this difference in disease severity between these two time periods, it cannot account for most of this improvement. Furthermore, our subjects' improvement was better than that described in historical studies before the introduction of immunosuppressant therapies.[36]

Figure 5 illustrates the symptoms of LAPMG patients during three time periods: return exam symptoms, initial symptoms, and maximal symptoms. The data obtained for the initial and maximal symptoms are historical, but the return exam symptoms are based on an evaluation after patients were found positive for the antibodies. Ptosis and diplopia are common symptoms and occur in the majority of LAPMG patients during the maximum symptoms period. They are present to a lesser extent during other time periods. Other prominent symptoms in LAPMG patients at maximum disease severity are proximal upper extremity (UE) and lower extremity (LE) weakness, speech, swallowing, chewing, and respiratory and neck weakness. More than half of the LAPMG patients (54.5%) had respiratory symptoms. In addition to the usual bulbar findings seen in MG, LAPMG patients had proximal LE and UE symptoms during other stages of the disease.

Physical exam findings showed ptosis, diplopia, and mild weakness of the orbicularis oculi in more than 45% of LAPMG patients. More than 20% of LAPMG patients had orbicularis oris, neck flexion,
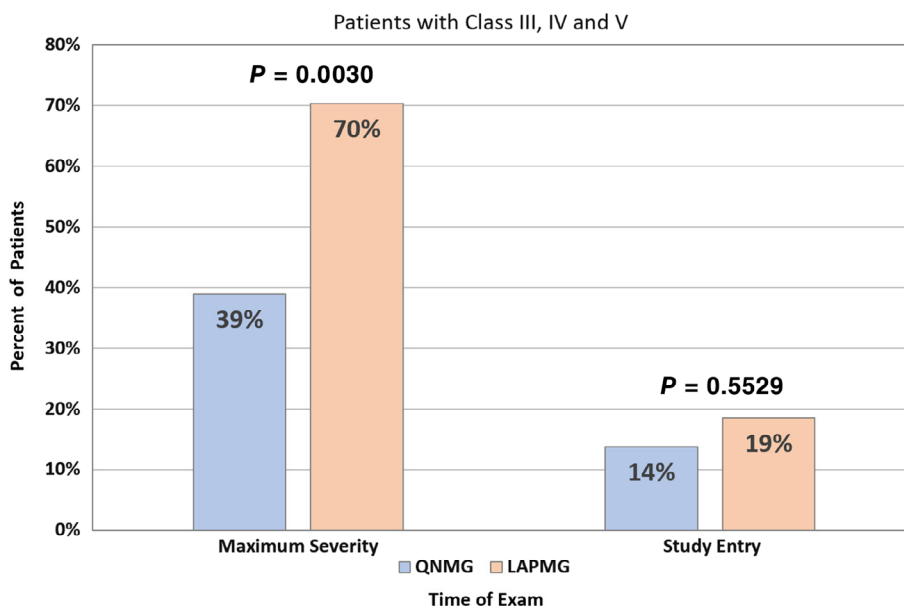

FIGURE 4 Percentage of patients with MGFA class III or higher at time of maximum disease severity and during the study entry exam. Abbreviations: MGFA, Myasthenia Gravis Foundation of America; QNMG, quadruple seronegative myasthenia gravis (negative for acetylcholine receptor, muscle-specific kinase, low-density lipoprotein receptor–related protein 4, and agrin) [Color figure can be viewed at wileyonlinelibrary.com]
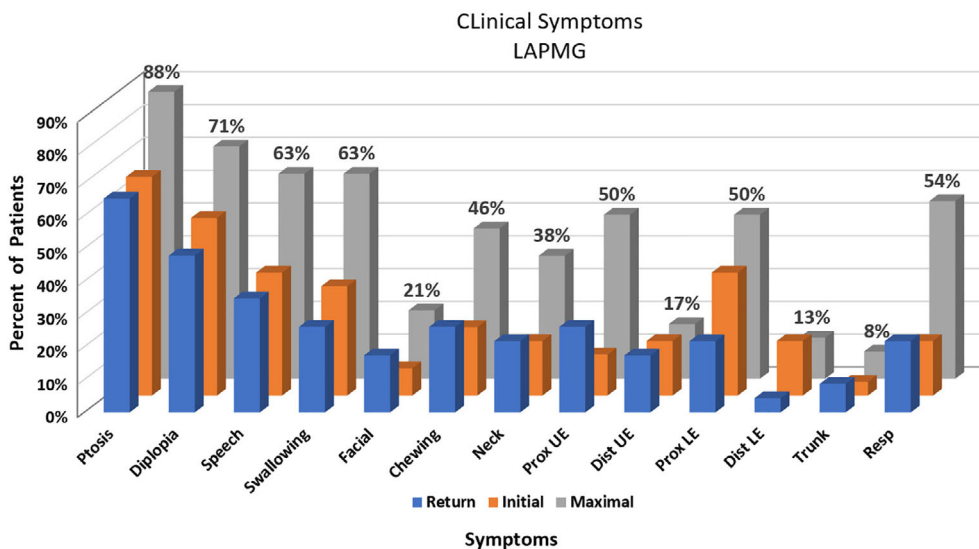
FIGURE 5 Clinical symptoms of LRP4/agrin-positive patients at three discrete time-points. Rear bars: maximal symptoms; middle bars: initial symptoms; front bars: return exam symptoms. Abbreviations: Dist, distal; LAPMG, double-seronegative myasthenia gravis with positivity for LRP4 and/or agrin antibodies; LRP4, low-density lipoprotein receptor–related protein 4; LE, lower extremity; Prox, proximal; Resp, respiratory; UE, upper extremity

shoulder, and hip weakness. The remainder of the exams, including reflex and sensory exams, were unremarkable. The average QMG score of LAPMG patients was 9.36 (SD, 5.54). Fifteen LAPMG patients (62%) had chest imaging. None had a thymoma. Five patients underwent a thymectomy before study entry. One patient had thymic hyperplasia; the remainder were normal.

4 | DISCUSSION

In this study we found that about 15% of DNMG patients had autoantibodies to either LRP4 or agrin. LRP4 autoantibodies were found in 13% of our double-negative patients, which coincides with the range of 2% to 50% reported in the literature.[10–12,25–30,32] and is in agreement with a previous report.[12] Agrin autoantibodies were found in 14% of our double-negative patients compared with the 0% to 50% reported in the literature.[13,14,26] Most (85%) of our LAPMG patients had autoantibodies to both agrin and LRP4. Because few patients in previous studies were tested for agrin, we do not know whether most of the other patients described in the literature were positive for both antibodies. Although the number of MG patients previously reported with agrin antibodies is too few to make any meaningful comparisons with our data (36 total and 5 with clinical data),[13,14,26] there are more data on LRP4 patients with MG (225 total and 133 with clinical data).[10–12,25–30,32] Some of the variability in incidence of LRP4 antibodies may be accounted for by the assay used. Several studies used cell-based assays,[11,25–29,32] and others, including ours, used ELISA[12–14,25] or alternative methods.[10] This cannot explain the varying incidences reported because different rates were reported using the same methods.

Ethnic or racial differences may account for some differing incidences. Lower incidences were reported in Asian populations[10,28,27]; however, in our population we found no difference among races, even though our study included relatively few minority patients. In addition, widely varying incidences were reported in two German studies.[11,25]

Several studies found incidences similar to ours.[29,32] The small numbers of patients studied may account for these disparities. We believe an incidence of around 15% reflects the likely proportion of positive patients in a group of DNMG patients, as this is similar to the rates seen in our work and the two largest studies.[29,33] It is of interest that most patients in our cohort who were positive for LRP4 were also positive for agrin. Although patients positive for both have been reported previously,[13] this is not a commonly observed finding among the few patients who have been tested for both antibodies.

Average age of onset (44 years) is consistent with that found in other studies.[11,29,32] Most LAPMG patients were female, also consistent with other studies.[11,27,29,32]

Our data on MGFA classification at symptom onset show that 31% of our LAPMG patients were MGFA class III or or higher. LAPMG patients were somewhat more symptomatic than those described by other studies at symptom onset, as most had fewer subjects with MGFA class III or higher and none had MGFA class V status.[25,32] A significantly greater percentage of our LAPMG patients had generalized MG at symptom onset compared with the QNMG group. At time of maximal severity, a significantly greater proportion of our LAPMG patients were MGFA class III or higher compared with our QNMG patients. Although some studies have shown only mild symptomatology with positive antibodies,[25,27–29,31,32] others have shown a more severe symptomatology, in agreement with our data.[10,11,24,33] Only the study of Zisimopoulou et al[32] included a sizable population of LRP4-positive patients. In their study, clinical data were collected on 67 DNMG patients who were LRP4-positive. Although both of our studies had a similar percentage of MGFA class I patients at disease onset, 31% of our LAPMG patients presented with MGFA class III or higher compared with 15% of their patients. One of our patients presented with MGFA class V, but none did so in the earlier study. The differences in the assay used may account for the differences between studies. The earlier study used a cell-based assay compared with the ELISA used in the present study. Also, the authors did not truly address maximum severity, whereas we had data on patients who had

an average disease duration of 11.3 years. Furthermore, the combination of antibodies to agrin and LRP4 may produce more severe disease than having antibodies only to LRP4. It is unknown whether the LRP4-positive patients in the Zisimopoulou et al[32] study were also positive for agrin antibodies because testing was not done. Only a single patient in our study was positive for LRP4 alone, and this patient had only mild symptoms. All three patients who were agrin-positive but LRP4 negative had maximum MGFA scores of III or higher, suggesting that presence of agrin antibodies is associated with more severe disease. At the time of maximum disease severity, a significantly greater number of LAPMG patients were MGFA class III or higher compared with QNMG patients. In fact, 11% of our LAPMG patients were ventilator-dependent as compared with 5% of our QNMG patients. Thus, we have shown that LAPMG patients have a more severe disease onset as well as a more severe course than QNMG patients.

On their return exam, LAPMG patients had mild ocular, oral, neck, proximal upper extremity, and proximal lower extremity symptoms and signs. Although the timing of the study entry exam was somewhat arbitrary, the percentage of LAPMG patients who were MGFA class III or higher was less at study entry exam (19%) compared with disease onset (31%) and point of maximal symptomatology (70%), which suggests that most LAPMG patients are optimally treated at the time of their study entry exam. The timing of the study entry exam in our comparison QNMG group was similarly arbitrary, as there was no significant difference in disease duration at the time of the study entry exam between the two groups. More than 40% of LAPMG patients had chewing, swallowing, speech, respiratory, neck, and proximal upper and lower extremity weakness at maximum severity. Although we did not obtain comparable data in our QNMG group, based on the difference in MGFA classification, we believe bulbar, respiratory, and limb symptoms are more common in LAPMG patients.

Despite having more severe symptoms initially and during the course of their disease, most LAPMG patients improved with standard immunosuppressive or immunomodulatory therapy. We do not have enough data to determine which therapies are most effective, and this is not a prospective, randomized therapeutic head-to-head comparative study, but the presence of these antibodies might guide future therapeutic choices. With the development of complement-modifying therapies such as eculizumab, identifying what antibodies are present in patients may allow us to tailor therapy and thus improve treatment.[37] Because presence of agrin and LRP4 antibodies is associated with a more severe disease course compared with having with no detectable autoantibodies, finding these antibodies in DNMG patients will allow us to better predict which patients will develop severe disease. According to the data we currently have, we could find no distinguishing clinical characteristics that would allow us to differentiate LAPMG from QNMG. More information will be needed to compare severity and clinical characteristics with those seen in AChR- and MuSK-positive MG. The current data show that many LAPMG patients have severe clinical courses, similar to those seen in many AChR- and MuSK-positive patients. As with all MG patients, current management can lead to clinical improvement in DNMG patients.[38]

We suggest ordering LRP4 and agrin antibodies in MG patients who are negative for AChR and MuSK antibodies (DNMG).

The final issue concerns whether these antibodies (LRP4/agrin) produce disease pathology or are just an epiphenomenon. Antibodies to LRP4 and agrin led to pathology in mice that mimics MG in humans.[15,16] Furthermore, inherited defects of LRP4 and agrin produce congenital MG.[18–23] Although presence of agrin and LRP4 antibodies has also been associated with amyotrophic lateral sclerosis,[39,40] LRP4 with neuromyelitis optica[12] and multiple sclerosis,[32] other disease control patients tested in the literature were antibody-negative. Based on the location of LRP4 at the neuromuscular junction, its binding to agrin, and its relationship to MuSK activation, it is highly likely that the presence of these antibodies in DNMG is related to their disease pathology.[6,7]

In conclusion, in this study we found that 15% of DNMG patients had antibodies to either LRP4 or agrin and 13% of our patients had autoantibodies to both. The average age of onset was 44 years and 59% were female. LAPMG patients have a more severe presentation compared with QNMG patients. The presence of these autoantibodies was associated with a more severe disease course, but most patients responded well to standard MG therapy.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

CONFLICT OF INTEREST

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APPENDIX

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