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Serological Markers of Clinical Improvement in MuSK Myasthenia Gravis

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Abstract

Background and Objectives

In this retrospective longitudinal study, we aimed at exploring the role of (a) MuSK-immunoglobulin G (IgG) levels, (b) predominant MuSK-IgG subclasses, and (c) antibody affinity as candidate biomarkers of severity and outcomes in MuSK-MG, using and comparing different antibody testing techniques.

Methods

Total MuSK-IgGs were quantified with radioimmunoassay (RIA), ELISA, flow cytometry, and cell-based assay (CBA) serial dilutions using HEK293 cells transfected with MuSK-eGFP. MuSK-IgG subclasses were measured by flow cytometry. SAffCon assay was used for determining MuSK-IgG affinity.

Results

Forty-three serum samples were obtained at different time points from 20 patients with MuSK-MG (median age at onset: 48 years, interquartile range = 27.5–72.5; women, 16/20), with 9 of 20 (45%) treated with rituximab. A strong correlation between MuSK-IgG levels measured by flow cytometry and RIA titers was found (r_s = 0.74, 95% CI 0.41–0.89, p = 0.0003), as well as a moderate correlation between CBA end-point titers and RIA titers (r_s = 0.47, 95% CI 0.01–0.77, p = 0.0414). A significant correlation was found between MuSK-IgG flow cytometry levels and disease severity (r_s = 0.39, 95% CI 0.06–0.64, p = 0.0175; mixed-effects model estimate: 2.296e-06, std. error: 1.024e-06, t = 2.243, p = 0.032). In individual patients, clinical improvement was associated with decrease in MuSK-IgG levels, as measured by either flow cytometry or CBA end-point titration. In all samples, MuSK-IgG4 was the most frequent isotype (mean ± SD: 90.95% ± 13.89). A significant reduction of MuSK-IgG4 and, to a lesser extent, of MuSK-IgG2, was seen in patients with favorable clinical outcomes. A similar trend was confirmed in the subgroup of rituximab-treated patients. In a single patient, MuSK-IgG affinity increased during symptom exacerbation (K_D values: 62 nM vs 0.6 nM) while total MuSK-IgG and IgG4 levels remained stable, suggesting that affinity maturation may be a driver of clinical worsening.

Discussion

Our data support the quantification of MuSK antibodies by flow cytometry. Through a multimodal investigational approach, we showed that total MuSK-IgG levels, MuSK-IgG4 and MuSK-IgG2 levels, and MuSK-IgG affinity may represent promising biomarkers of disease outcomes in MuSK-MG.

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Glossary

CBA = cell-based assay; **ECD** = extracellular domain; **FACS** = fluorescence-activated cell sorting; **IgG** = immunoglobulin G; **IQR** = interquartile range; **MG** = myasthenia gravis; **PBS** = phosphate-buffered saline; **PIS** = postintervention status; **RIA** = radioimmunoassay; **RTX** = rituximab; **ΔMFI** = delta median fluorescent intensity.

Introduction

Myasthenia gravis (MG) with antibodies against the muscle-specific tyrosine kinase (MuSK-MG) is a rare disease, usually characterized by predominant bulbar muscle weakness.[1] MuSK antibodies are mostly immunoglobulin G4 (IgG4) and are believed to be produced almost exclusively by short-lived plasma cells, which continuously differentiate from $CD20^+$ memory B-cell precursors.[2-4] In the past decade, there has been a significant improvement in the clinical outcome of MuSK-MG, due to earlier recognition and improved treatment.[5] In particular, rituximab (RTX) is generally believed to be very effective,[4,6,7] inducing prolonged remissions associated with reduction of serum antibody levels.[2,8,9] In some patients, however, sustained clinical response coexists with detectable antibody levels.[10] In these cases, the clinical improvement after RTX may reflect a modification of the B-cell repertoire, a switch in the predominant MuSK-IgG subclass,[2,11] or a shift in the antibody specificity (from relevant to irrelevant epitopes, e.g., those located on the cytoplasmatic domain that are not accessible to circulating antibodies). In this study, different antibody assays were used and compared, with the aim of exploring the role of (a) MuSK-IgG levels, (b) predominant MuSK-IgG subclasses, and (c) antibody affinity as candidate biomarkers of clinical outcomes in a longitudinal cohort of patients with MuSK-MG.

Methods

Patient Enrollment

This retrospective longitudinal study included patients with MuSK-MG treated at Policlinico Universitario "A. Gemelli" IRCCS (Rome, Italy) between 2000 and 2021, from whom at least 2 serum samples collected at different time points were available. All the patients were diagnosed and treated according to standard clinical practice[12] and tested positive for MuSK antibodies by radioimmunoassay (RIA) (RSR Limited, Cardiff, UK) at diagnosis. Clinical severity was classified following the Myasthenia Gravis Foundation of America (MGFA) clinical classification, which subgroups patients with signs/symptoms of MG into 5 severity classes (purely ocular MG as class I; mild, moderate, and severe generalized MG as classes II, III, and IV, respectively; and MG crisis as class V).[13] The MG outcome was classified with the MGFA postintervention status (PIS).[13] Clinical information was available for all serum samples. All these patients had generalized MG, MGFA grades II-V at maximal disease severity. For each patient, at least 1 serum sample was obtained during an acute symptomatic phase of the disease, either at disease onset or during MG exacerbation. In each patient, the samples obtained during the acute phase were compared with the one(s) after therapeutic intervention. The favorable outcome was defined by a MGFA PIS of "minimal manifestation (MMs)–or-better."

Cell-Based Assays for MuSK Antibodies

In-house live cell-based assay (CBA) for MuSK antibody detection was performed as previously reported.[14] In brief, HEK293 cells were transiently transfected with polyethyleneimine (PEI, cat#: 408727, Sigma-Aldrich, MO) with a plasmid encoding for MuSK-eGFP (kindly provided by Prof. Beeson and Prof. Vincent, Oxford, UK) and, 36–48 hours after transfection, were incubated with patients' serum samples (1:20 dilution) for 1 hour at room temperature. After fixation with 4% paraformaldehyde (PFA, cat# 158127, Sigma-Aldrich), human IgG labeling was demonstrated with a secondary goat anti-human IgG Fc-specific Alexa Fluor 594 antibody (1:750, 09-585-098, Jackson Labs). Coverslips were then mounted with a mounting medium (DakoCytomation, Cambridge, UK) containing 4′,6-diamidino-2-phenylindol (DAPI, 1:1000). CBA results were assessed by fluorescence microscopy on the following day, and labeling of the secondary antibody was scored as previously described.[15] Antibody titration was performed using 2-fold serial dilutions (starting at 1:25), the last one giving detectable signal (CBA score ≥1) being considered as endpoint dilution.

Quantification of Total MuSK-IgG and MuSK-IgG Subclasses by Flow Cytometry

Serum IgG labeling on MuSK-transfected live HEK293 cells was quantified using fluorescence-activated cell sorting (FACS) analysis, as previously described.[16] In brief, all serum samples (1:20 dilution in FACS buffer: phosphate-buffered saline (PBS) 1X, cat# 10010023, Gibco-Thermo Fisher Scientific, USA; 0.1% bovine serum albumin, Sigma-Aldrich, USA; 2 mM EDTA, cat# E6758, Sigma-Aldrich) were incubated for 1 hour with 100,000 MuSK-eGFP HEK293 cells in suspension; then, after washing, IgG binding was detected with a mouse anti-human IgG Alexa Fluor 647 secondary antibody (1:400, cat#: 9040-31, Southern Biotech). Finally, cells were stained with DAPI (1:1000) for 15 minutes and then assessed by FACS (CytoFLEX, Beckman Coulter Life Sciences). The levels of each MuSK-IgG isotype were determined using the same protocol and the following secondary antibodies: mouse anti-human IgG1 Alexa Fluor 647 (1:200, cat#: 9052-31, Southern Biotech, USA), mouse anti-human IgG2 Alexa Fluor 647 (1:200, cat#: 9070-31, Southern Biotech), mouse anti-human IgG3 Alexa Fluor 647 (1:200, cat#:9210-31, Southern Biotech), and mouse anti-human IgG4 PE (1:400, cat#: 9200-09, Southern Biotech). Total

MuSK-IgG levels and MuSK-IgG subclasses were calculated by the delta median fluorescent intensity (ΔMFI) of the transfected (single cells/viable/eGFP-positive gates) minus untransfected (single cells/viable/eGFP-negative gates) cells. The cutoff was determined for each ΔMFI using 10 healthy controls (mean value plus 3 standard deviations). To quantify antibody binding and standardize ΔMFI values across different experimental sessions, Quantum Simply Cellular (QSC) anti-mouse IgG beads (Cat.# 815, Bangs Laboratories, Inc) were used according to the manufacturer's instructions. Each QSC kit consists of 5 bead populations—1 blank and 4 populations with increasing levels of Fc-specific capture antibody. QSC microspheres labeled with fluorochrome-conjugated antibodies have the same spectral properties as cells labeled with the same antibodies; they thus serve as an accurate compensation standard that covers the intensity range of cells with different expression levels. In brief, QSC beads were labeled to saturation with the same antibody that was used to label cells (diluted in FACS buffer) and run on the same instrument, using the same settings and on the same day as cell samples to reduce any possible bias. Channel values for the bead populations were recorded in lot-specific QuickCal software, and a regression associating fluorescence channel values to the beads' antibody binding capacity (ABC) values was calculated. ABC values were then assigned to stained cell samples using this standard curve. Through this method, for each sample, ΔMFI was converted into its corresponding standardized ABC value, which was used to quantify the amount of antibodies bound to MuSK-HEK293 cells.

Recombinant MuSK Protein Generation

The extracellular domain (ECD) of human MuSK (UniProt position: 1–472) was subcloned into the pcDNA3.1 vector. The C-terminal region contained a short, flexible linker, followed by a 6× histidine tag. HEK293F cells were transduced using linear PEI at a vector-to-PEI ratio of 1:3, and culture supernatants were harvested 3 days after transduction. The recombinant MuSK ECD was purified using HisPur Ni-NTA beads (Thermo Fisher Scientific) as per the manufacturer's instructions.

Measurement of MuSK-IgG by Enzyme-Linked Immunosorbent Assay (ELISA)

Binding strength of serum to recombinant MuSK ECD was assessed by direct ELISA. Recombinant MuSK was coated on Nunc MaxiSorp ELISA plates (Biolegend) at a concentration of 2 μg/mL overnight at 4°C and subsequently blocked with blocking buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0). Serial dilutions of the serum were prepared in sample buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0). After 3 washes of plates with wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), 100 μL of each serial dilution was added to the plates in duplicate and incubated for 1 hour. Plates were then washed 3 times with 250 μL per well of wash buffer. For detection of IgG binding, 1:3000 dilution of a goat anti-human IgG-horseradish peroxidase–conjugated secondary antibody (Thermo Fisher Scientific) was prepared in a sample buffer and 100 μL was added to each well for 1 hour. Plates were washed further 3 times and developed for 10 minutes with 1-Step TMB ELISA Substrate (Thermo Fisher Scientific), and reaction was stopped by the addition of 3M hydrochloric acid. The optical density at 490 nm (OD490) was measured using a FLUOstar Omega Microplate Reader (BMG Labtech). The background value was set at the OD490 value of blank wells without incubation of serum samples, and the area under the curve was calculated. Data were analyzed in R stats.[17]

Titration of MuSK Antibodies by RIA

To quantify the antibodies, each sample was first serially titrated to establish the volume of serum required to immunoprecipitate 50% of the available ^{125}I-MuSK (approx. 10,000 cpm; RSR Ltd, United Kingdom). The final volumes varied from 0.003 μL to 0.1 μL, indicating levels of MuSK antibodies between 12 and 360 nM. The values are given in eTable 1.

Determination of MuSK Antibody Affinity by SAffCon Assay

To determine affinity and concentration, in a subset of samples with enough material available, dilutions of serum (1:100, 1:20, 1:5) were mixed with Alexa Fluor 647 labeled MuSK protein (10 nM and 100 nM) and incubated for 30 minutes at 4°C to allow equilibration of the binding interaction. Samples were run in duplicate on a Fluidity One-M instrument (Fluidic Analytics) using size setting 3 (3.0–14 nm) and viscosity setting 3 to determine the average hydrodynamic radius (Rh) of the labeled species in the mixture. The flow buffer used was PBS (pH 7.4) with 5% (w/v) human serum albumin and 10% (w/v) glycerol. Data were fit using SAffCon with Bayesian fitting application of Fluidity Intelligence, which determined initial fit parameters and additional points that would most efficiently improve fit quality. Additional points were run as recommended and added to the analysis in an iterative manner until results converged or no further improvement could be achieved.[18] To confirm that the measurements provided reflect antibody affinity and not avidity, raw data were checked to rule out evidence of network formation that could result from polyclonal antibodies cross-linking MuSK by binding at different epitopes (which would lead to the measurement of avidity using a solution-phase technique as the one used here). No evidence of network formation was found in any of the analyzed samples.

Statistical Analysis

Continuous variables are presented as median with interquartile range (IQR) and categorical variables as proportions and percentages. Data distribution was assessed using the Shapiro-Wilk normality test. Statistical comparisons were performed using the Student *t* test for paired data or the Wilcoxon signed rank test, as appropriate. Correlation analysis was performed using a linear mixed-effects model or Spearman correlation, as appropriate. The linear mixed-effects model takes into account multiple measurements per

Table 1 Patients' Demographic and Clinical Characteristics

Female sex, n (%)	16 (80)
Median age at disease onset, y (IQR)	48 (27.5–72.5)
Median follow-up at the last study visit, mo (IQR)	50 (13–100.5)
Disease severity (max. MGFA class), n (%)	
IIb	1 (5)
IIIb	10 (50)
V	9 (45)
Median time from onset to immunotherapy, mo (IQR)	2 (0–10)
Immunotherapy, n (%)	
Corticosteroids only	4 (20)
Corticosteroids + 1 immunosuppressant[a]	6 (30)
Corticosteroids + RTX	3 (15)
Corticosteroids + 1 immunosuppressant[b]+ RTX	6 (30)
1 immunosuppressant only[c]	1 (5)
Outcome (MGFA PIS at the last study follow-up visit), n (%)	
CSR	1 (5)
PR	4 (20)
MM	5 (25)
I	4 (15)
U	4 (25)
W	2 (10)

Abbreviations: CSR = complete stable remission; I = improved; IQR = interquartile range; MG = myasthenia gravis; MGFA = Myasthenia Gravis Foundation of America; MMs = minimal manifestations; PR = pharmacologic remission; U = unchanged; W = worse.
[a] Azathioprine, 4 patients; mycophenolate mofetil, 2 patients.
[b] Mycophenolate mofetil, 4 patients; azathioprine 1; cyclosporine 1.
[c] Methotrexate, 1 patient.

patient and the variability in the number of measurements between patients. The strength of the correlation was quantified with the Spearman rank correlation coefficient (r_s). To depict the relationship between variables, a simple linear regression was performed. A p value inferior to 0.05 was considered statistically significant. Statistical analysis and graphs were done with Graph Pad Prism v10 (Graph Pad Software, La Jolla, CA) and R stats.[17]

Standard Protocol Approvals, Registrations, and Patient Consents

All patients provided written consent to the participation in this study. The study was conducted according to the Helsinki Declaration and approved by the Ethics Committee of the Catholic University of Sacred Heart (Rome, Italy) with E.C. protocol number 49886/18 (9024/19).

Data Availability

Anonymized data not published within this article will be made available on reasonable request from any qualified investigator.

Results

Patient Cohort

This study included 20 adult patients with MuSK-MG, 16 of 20 women (80%), with a median age at disease onset of 48 years (IQR: 27.5–72.5) and a median follow-up at the last study visit of 50 months (IQR: 13–100) (Table 1). Overall, the disease course was moderate to severe, with 9 of 20 patients (45%) having experienced a MG crisis during the disease course and a single case with mild disease (MGFA at maximum severity = II). At the last study visit, 10 of 20 patients (50%) had achieved a status of MM-or-better and 4 of 20 (20%) were improved compared with baseline (but still symptomatic) while the remaining were either unchanged (4/20, 20%) or worsened (2/20, 10%). RTX was administered to 9 of 20 patients (45%) at a median (IQR) time of 10 (2.5–15) years after disease onset (eTable 2). These patients were more severe compared with non-RTX patients (max. MGFA = V in 7/9 vs 2/11 patients, $p = 0.0216$). None of the patients received IVIg, in line with the known poor response of MuSK-MG and other IgG4-mediated disorders to such treatment.[4]

In total, 43 serum samples were collected at different time points during the disease course (eTable 3). Pretreatment samples were available in 7 of 20 patients (35%). 14 of 43 serum samples (33%) were obtained from patients with MM-or-better status and 29 of 43 (67%) during a symptomatic phase (MGFA class at sampling: IIA in 1 patient, IIb in 12, IIIa in 1, IIIb in 14, and IVb in 1).

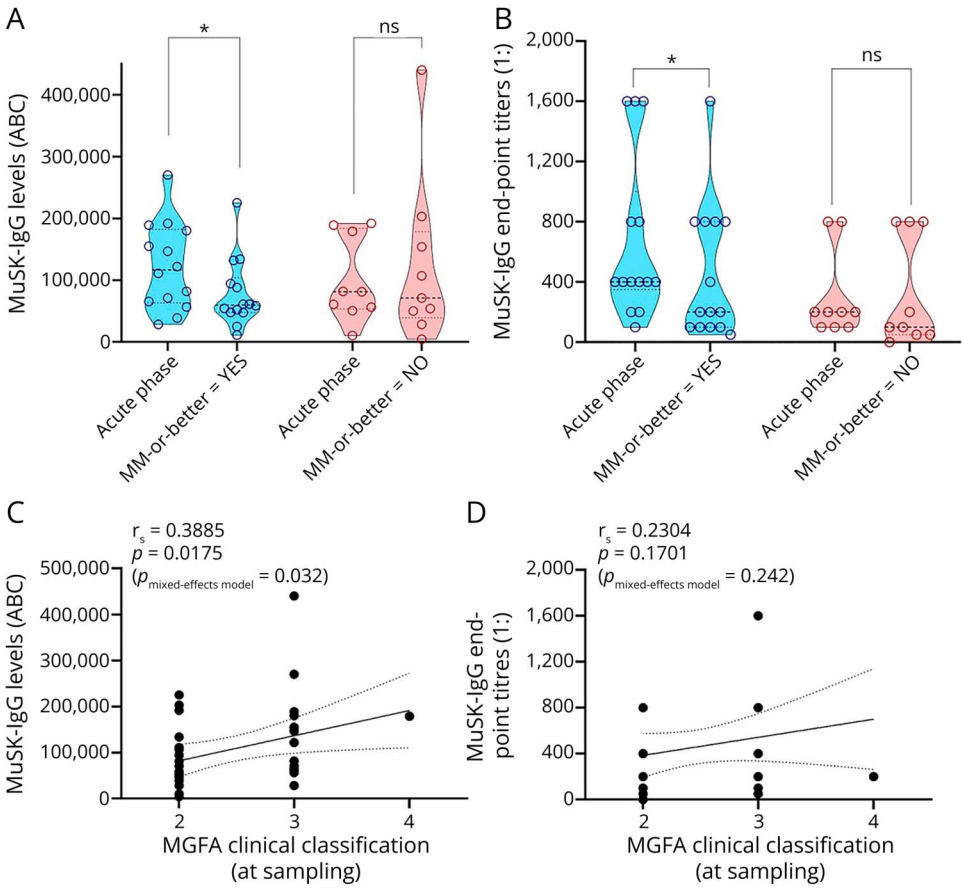
Quantification of MuSK-IgG Levels: Correlation Between Antibody Assays

Total MuSK-IgG levels were measured using FACS and CBA serial dilutions (ABC levels and end-point titers, respectively) in all samples. In a subgroup of serum samples with enough available material, MuSK-IgG was also quantified by ELISA (n = 23) and/or by RIA (n = 19). We found a strong positive correlation between MuSK-IgG levels measured by FACS and by RIA ($r_s = 0.7346$, $p = 0.0003$) (eFigure 1A), a moderate positive correlation between CBA end-point titers and RIA titers ($r_s = 0.4718$, $p = 0.0414$) (eFigure 1B), and no correlation between CBA end-point titers and MuSK-IgG levels measured by FACS (eFigure 1C). Finally, no correlation was found between ELISA and MuSK-IgG ABC, end-point titers, and RIA titers (eFigure 1, D–F).

Anti-MuSK-IgG Levels and Correlation With Disease Status and Outcomes

We assessed the correlation between MuSK-IgG levels measured by FACS, end-point CBA titers, and clinical status. A summary of clinical and serologic features for each patient is

Figure 1 MuSK-IgG Levels, End-Point Titers, and Clinical Status



Comparison of MuSK-IgG levels (antigen binding capacity, ABC) in serum samples collected from patients during an acute MG phase and at a second time point, when they either achieved a favorable (MGFA PIS: MM-or-better = YES) unfavorable (MGFA PIS: MM-or-better = NO) outcome. (A) A reduction of MuSK-IgG levels was found in patients who achieved a favorable clinical outcome (p = 0.0295), with no significant changes in those who did not. (B) A decrease in MuSK-IgG CBA end-point titers was associated with the achievement of an MGFA PIS of MM-or-better (p = 0.0156) while no significant changes were observed in patients who were still symptomatic after immunotherapy. (C) Mixed-effects modeling showed a significant positive correlation between MuSK-IgG levels (ABC) and clinical severity classified according to the MGFA clinical classification (r_s = 0.3885, 95% CI 0.06383–0,6387, p = 0.0175; mixed-effects model estimate: 2.296e-06, std. error: 1.024e-06, t = 2.243, p = 0.032) (a simple linear regression line is plotted; dotted lines show 95% CI). (D) Mixed-effects modeling did not show a significant correlation between MuSK-IgG CBA end-point titers and clinical severity classified according to the MGFA clinical classification (r_s = 0.2304, 95% CI-0.1110-0.5232, p = 0.1701; mixed-effects model estimate: 2.474e-04, std. error: 2.077e-04, t = 1.191, p = 0.242) (a simple linear regression line is plotted; dotted lines show 95% CI). MGFA = Myasthenia Gravis Foundation of America; MM = minimal manifestation; PIS = postintervention status.

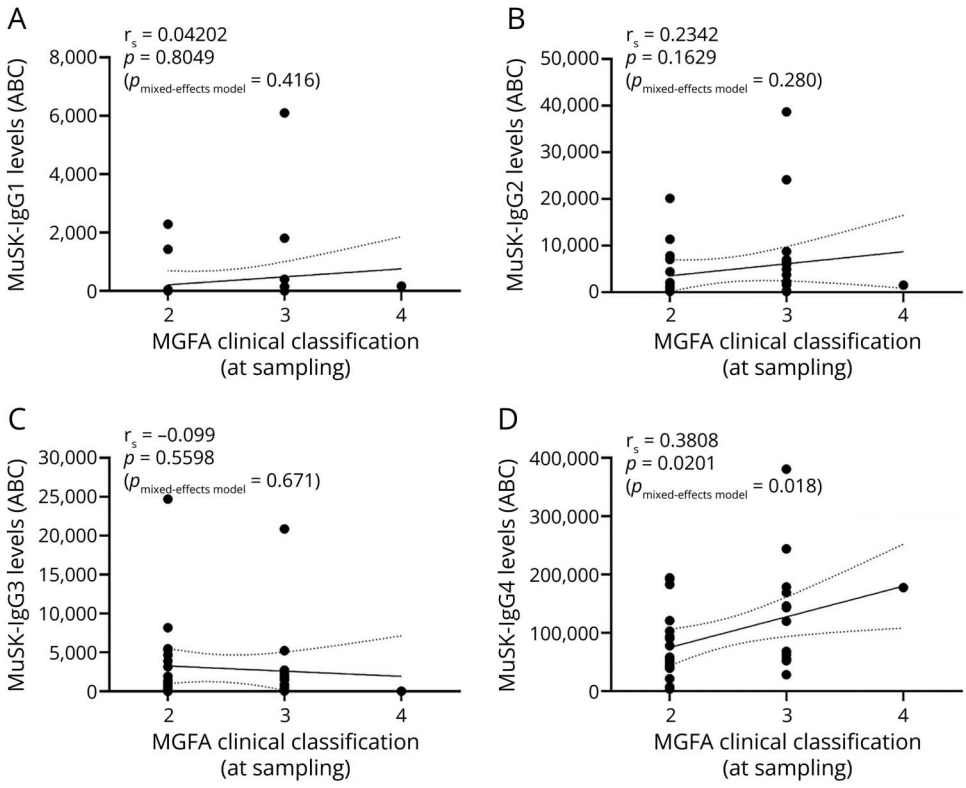
provided in eTable 1. Achievement of a MGFA PIS of MM-or-better was associated with a decrease in MuSK-IgG levels (median: 59,853, IQR: 47,826–94,533) compared with the acute symptomatic phase (median: 116,889, IQR: 65,478–18,034) as assessed by FACS (p = 0.0295) (Figure 1A). In these patients, median MuSK-IgG end-point titers by live CBA also decreased (1:200 vs 1:400, p = 0.0156) (Figure 1B). Mixed-effects modeling revealed a significant positive correlation between MGFA clinical classification at sampling and total MuSK-IgG levels measured by FACS (r_s = 0.3885, 95% CI 0.06383–0,6387, p = 0.0175; mixed-effects model estimate: 2.296e-06, std. error: 1.024e-06, t = 2.243, p = 0.032) (Figure 1C), but not with CBA end-point titers (r_s = 0.2304, 0.1110–0.5232, p = 0.1701; mixed-effects model estimate: 2.474e-04, std. error: 2.077e-04, t = 1.191, p = 0.242) (Figure 1D). When considering only RTX-treated patients, clinical improvement was paralleled by a trend toward a reduction of both total MuSK-IgG ABC levels (median of ABC differences: −107000, −65%; p = 0.0625) and median MuSK-IgG end-point titers (1:100 vs 1:400, p = 0.0625) (eFigure 2, A and B). Overall, these data show that both MuSK-IgG ABC levels and end-point titers significantly decreased in patients who achieved a good clinical outcome as defined by the MGFA-PIS classification.

Correlation of MuSK Subclass Levels With Disease Status and Outcomes

In all samples, MuSK-IgG4 was the most frequent isotype, representing on average (±SD) 90.95% ± 13.89 of MuSK antibodies, followed by IgG3 (3.29% ± 4.52) and IgG2 (3.27% ± 3.98), with IgG1 being the least frequent subclass (2.47% ± 13.04). MuSK-IgG4 was detected in all samples, IgG3 in 42 of 43 (97.67%), IgG2 in 38 of 43 (88.37%), and IgG1 in 15 of 43 (34.88%). In longitudinal samples, MuSK-IgG4 was the predominant subclass (>50% of total MuSK-IgG) in all patients sampled during the acute phase and remained the dominant isotype irrespective of disease outcomes, except for 1 patient, who switched to IgG1 when in pharmacologic remission (acute phase: IgG4 95.66%, IgG1 2.76%; pharmacologic remission: IgG4 13.73%, IgG1 85.45%). Of interest, in this patient, total MuSK-IgG levels (ABC) did not significantly decrease (ABC values: 6.55×10^4 vs 2.50×10^4) while MuSK-IgG CBA end-point titers increased from 1:400 to 1:800, pointing to IgG class switch as the main immunobiological mechanism driving clinical remission.

A significant positive correlation was found between MGFA scores and MuSK-IgG4 levels by FACS (r_s = 0.3808, 95% CI 0.05487–0.6334, p = 0.0201; mixed-effects model estimate: 2.740e-06, std. error: 1.129e-06, t = 2.426, p = 0.018) while no

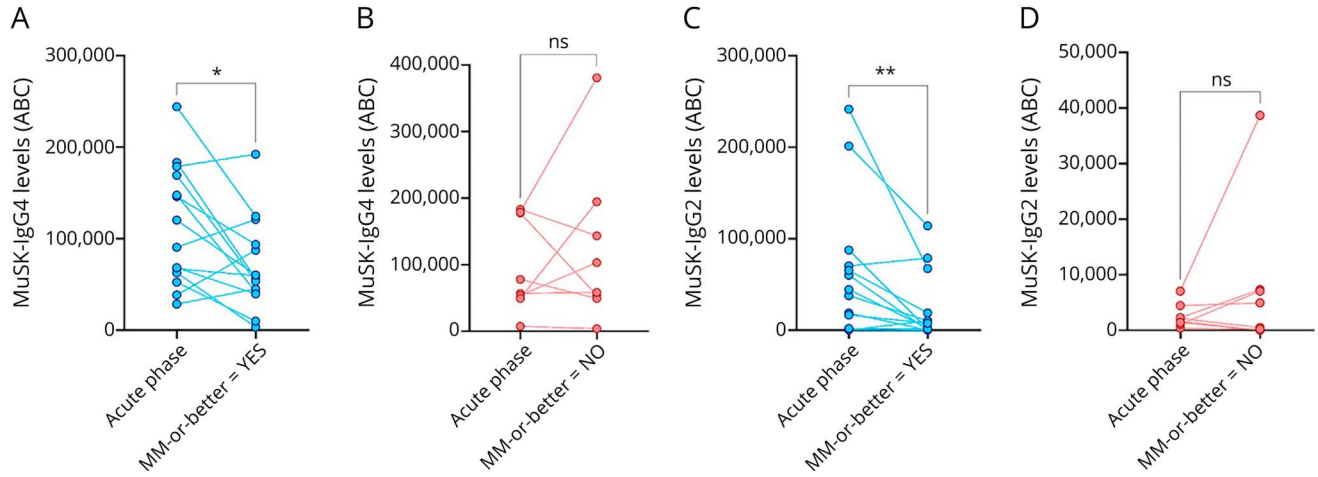
Figure 2 MuSK-IgG Subclasses and Disease Severity



Scatter plots showing MuSK-IgG subclass levels (ABC) measured by flow cytometry live CBA and disease severity assessed by MGFA classification at sampling. Mixed-effects modeling revealed no significant correlations between disease severity and the levels of MuSK-IgG1 (r_s = 0.04202, 95% CI-0.295-0.3697, p = 0.8049; mixed-effects model estimate: 6.970e-05, std. error: 8.458e-05, t = 0.824, p = 0.416) (A), MuSK-IgG2 (r_s = 0.2342, 95% CI −0.1070 to 0.5261, p = 0.1629; mixed-effects model estimate: 1.290e-05, std. error: 1.175e-05, t = 1.097, p = 0.280) (B), and MuSK-IgG3 levels (r_s = −0.099, 95% CI −0.4181 to 0.2418, p = 0.5598; mixed-effects model estimate: −7.752e-06, std. error: 1.814e-05, t = −0.427, p = 0.671) (C) and a significant positive correlation with MuSK-IgG4 levels (r_s = 0.3808, 95% CI 0.05487–0.6334, p = 0.0201; mixed-effects model estimate: 2.740e-06, std. error: 1.129e-06, t = 2.426, p = 0.018) (D). A simple linear regression line is plotted; dotted lines show 95% CI. MGFA = Myasthenia Gravis Foundation of America.

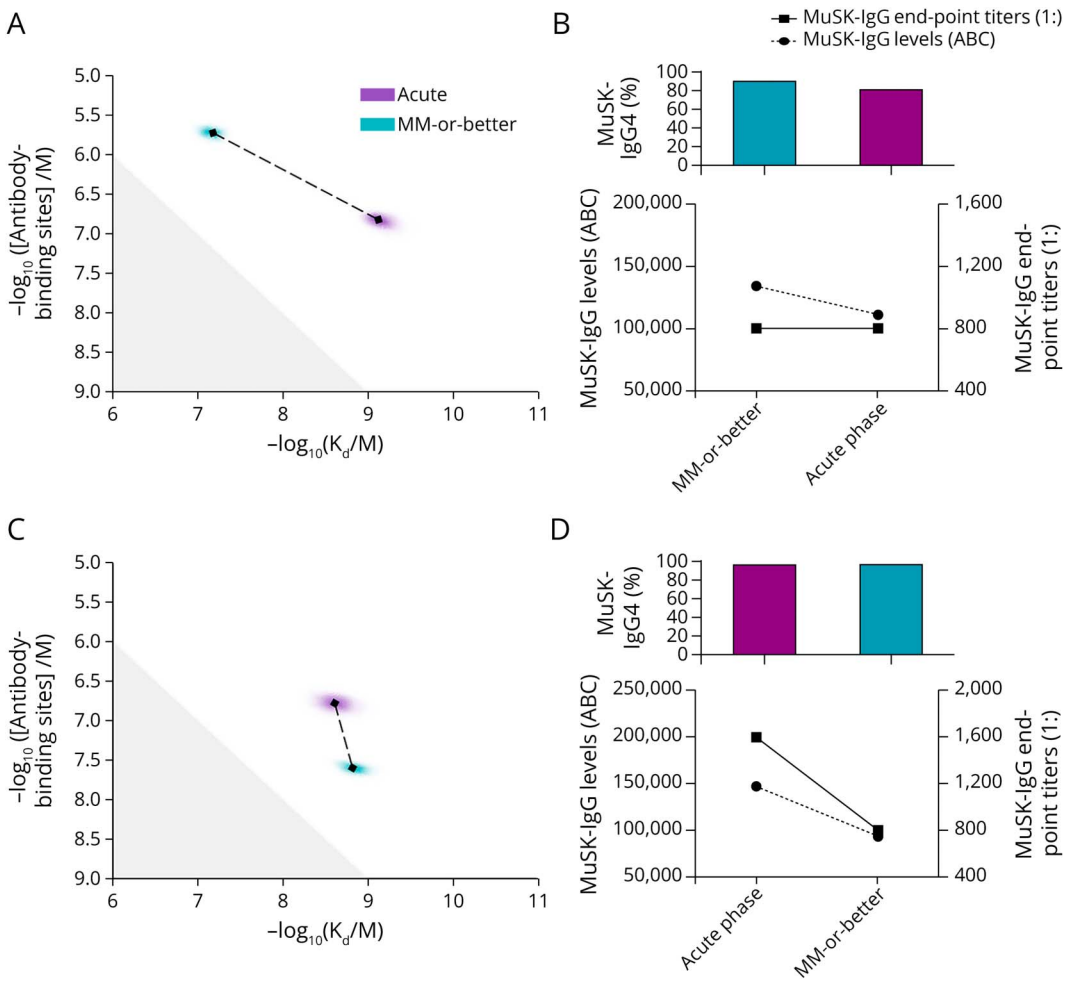
significant correlations between disease severity and MuSK-IgG1-3 levels were found (Figure 2, A–D). We observed a significant reduction of both IgG4 (median of ABC differences = −47,438; p = 0.0245, Figure 3A) and IgG2 (median of ABC differences = −2,303; p = 0.0085, Figure 2C) in samples obtained in MM-or-better status compared with acute symptomatic phases. Of interest, in 4 patients, MM-or-better was achieved despite stable (or slightly increased) MuSK-IgG4 levels. In one of these patients (Pt.#5), a reduction of MuSK-IgG2 paralleled the achievement of MM-or-better

Figure 3 MuSK-IgG Subclasses and Clinical Outcomes



(A) MuSK-IgG4 reduction was associated with achievement of a favorable clinical outcome (p = 0.0245). (B) No significant changes in MuSK-IgG4 levels were observed in patients who were still symptomatic after immunotherapy (p = 0.7422). (C) Pairwise comparison of MuSK-IgG2 levels show a significant reduction in patients who achieved an MGFA PIS of MM-or-better (p = 0.0085). (D) No significant changes in MuSK-IgG2 levels were observed in patients with an unfavorable clinical outcome (p = 0.5469). MGFA = Myasthenia Gravis Foundation of America; PIS = postintervention status.

Figure 4 MuSK-IgG Affinity and Clinical Status



(A) Affinity-concentration plot showing SAffCon assay results in longitudinal samples from the same patient, who had an MGFA PIS of MM-or-better at time of initial sampling, with antibody properties of K_D = 62 nM and concentration (antibody-binding sites) = 1,900 nM. The second serum sample was collected during a phase of clinical exacerbation (classified as "acute phase"), with antibodies showing a 100-fold increase in affinity (K_D = 0.6 nM) and a concentration = 140 nM. The K_D is plotted on the x-axis as –log10(M); thus, higher values correspond to lower K_D (and hence tighter binding). Antibody concentration is plotted on the y-axis as –log10(M); thus, lower values correspond to higher antibody concentrations. (B) Total MuSK-IgG levels (ABC), CBA end-point titers, and proportion of specific IgG4 of the same patient and time points showed in panel A, demonstrating the same end-point titers, a mild decrease in MuSK-IgG levels, and predominance of IgG4 at both time points (90.8% and 81.3%, respectively). (C) Affinity-concentration plot showing SAffCon assay results in longitudinal serum specimens of another patient, who was sampled during the acute MG phase at the first time point, with antibody properties of K_D = 2 nM and concentration = 160 nM. At the second time point, when a favorable clinical outcome was achieved (MGFA PIS: MM-or-better) after immunotherapy, the antibody properties were K_D = 1.3 nM and concentration = 24 nM. K_D and antibody concentrations are plotted as described above. (D) Total MuSK-IgG levels (ABC), CBA end-point titers, and proportion of specific IgG4 of the same patient and time points showed in panel C, demonstrating a reduction of both MuSK-IgG levels (ABC) and CBA end-point titers while the predominant MuSK-IgG subclass was IgG4 at both time points (99.6% and 99.1%, respectively). MGFA = Myasthenia Gravis Foundation of America; PIS = postintervention status.

while in the other patient (Pt.#3), lower MuSK antibody affinity was measured in the sample obtained during symptom remission (Figure 4, A and B; described in more detail in the following section). Conversely, no significant changes of MuSK-IgG2 and IgG4 levels were found in patients who did not improve after treatment (median of ABC differences = +156.7 and −1,074, p = 0.5469 and p = 0.7422, respectively)—both in the total cohort (Figure 2, B and D) and in patients treated with RTX (eFigure 2, D and F). When considering only RTX-treated patients, a statistical trend toward a reduction of both MuSK-IgG4 and MuSK-IgG2 levels was observed only in treatment responders (median of ABC differences = −4,652 and −105,683, respectively, p = 0.0625) (eFigure 2, C and E).

MuSK-IgG Affinity and Correlation With Disease Status and Outcomes

We assessed the polyclonal MuSK-IgG affinity, using the SAffCon assay, in 25 samples from 13 patients. Antibody affinity quantification was possible in only 8 of 25 serum samples (32%), with a median K_D of 1.625 nM (range: 0.04 nM–61.9 nM), the remaining likely having K_D in picomolar range (very high affinity), less than the instrument quantitation threshold. In 2 patients, K_D values were available from paired samples obtained in acute symptomatic phase and during symptom remission (Figure 4, A and C). Notably, in 1 patient (Figure 4, A and B), both samples were obtained when the patient was not under immunosuppressive treatment. In

this patient, when considering the K_D alone, there was a 100-fold increase in MuSK antibody affinity during MG exacerbation (K_D values: 62 nM vs 0.6 nM). Of interest, MuSK-IgG levels remained substantially unchanged (ABC values: 1.34 x 10^5 vs 1.11 x 10^5), as well as CBA end-point titers (1:800 at both time points), as did the predominant IgG subclass (IgG4 90.8% and 81.3%) (Figure 4B). In the other patient (Figure 4, C and D), achievement of MM-or-better status was paralleled by a decrease in MuSK-IgG levels (ABC values: 9.45×10^4 vs 1.47×10^5) and end-point titers (1:800 vs 1:1600) while antibody affinity remained substantially unchanged (K_D 1.3 nM vs 2 nM).

Discussion

In this study, we assessed the correlation between MuSK-MG clinical status and pathogenic antibodies' serum level, IgG subclasses, and affinity to find candidate biomarkers of disease severity and outcomes. We found that total MuSK-IgG and MuSK-IgG4 levels correlate with disease severity at the interindividual level when quantified by FACS. In addition, in longitudinal samples from individual patients, their reduction, along with the reduction of MuSK-IgG2, was associated with a favorable response to treatment, potentially representing a novel clinical biomarker that could help guide treatment choices.

Our results are in line with previous studies, which found a good correlation of MuSK antibody titers measured by RIA or ELISA with clinical severity and outcomes.[10,11,19] Of interest, similar findings were observed with antibodies targeting the MuSK Ig1 domain by ELISA, levels of which correlated with disease status intraindividually and interindividually.[20] Antibody detection by FACS is a high-throughput technique that provides quantitative and highly sensitive results. We here demonstrate that the measurement of MuSK-IgG levels by FACS strongly correlates with RIA antibody titers, confirming previous results,[21] whereas no correlation was found with ELISA titers. This finding is possibly explained by the non-native presentation of MuSK in absence of its transmembrane domain or the induction of conformational changes due to the adsorption of MuSK on ELISA plates. On the contrary, measurement of antibody levels by CBA end-point titration showed only a weak correlation with clinical severity, in line with the semiquantitative nature of this assay. Our data also showed that, within the same patient, MuSK-IgG levels decreased paralleling clinical improvement, either as measured by FACS or using CBA end-point titers. Similar findings were confirmed in RTX-treated patients. Altogether, these data support the FACS-based quantification of MuSK antibody levels as an efficient screening tool for diagnosing and monitoring MuSK-MG and a valuable single tool to screen and monitor all patients with MG. Owing to the observed discrepancies between FACS and ELISA MuSK antibody quantification, we caution the use of ELISA.

When assessing MuSK-specific IgG isotypes, we found that MuSK-IgG4 levels correlate with disease severity, in line with previous reports.[11] Of interest, when assessing response to conventional treatment, a reduction not only of MuSK-IgG4 but also of MuSK-IgG2 was associated with achievement of a favorable clinical outcome, a novel finding that requires further investigation. The lack of correlation between IgG2 levels and disease severity, despite its association with disease outcomes, could be due to both the relatively low frequency of MuSK-IgG2 and the use of different clinical scales to define severity and outcomes. Quantification of MuSK-IgG2 levels in larger samples of symptomatic patients is needed to clarify this point and to definitively prove the potential clinical usefulness of this serologic parameter. A trend toward a reduction of MuSK-IgG4 and MuSK-IgG2 was also observed in the subgroup of RTX-treated patients, further confirming that common immunobiological mechanisms underlie clinical improvement in most cases, irrespective of the treatment. This subanalysis likely did not reach statistical significance due to the low sample size; thus, validation of these findings in a larger patient cohort is needed. A switch in the predominant MuSK antibody subclass from IgG4 to IgG1 in association with the achievement of clinical remission has been so far reported in 2 patients.[2,11] In our cohort, we observed a similar event in 1 patient who switched from IgG4 to IgG1 when achieving symptom remission while total MuSK-IgG and MuSK-IgG4 levels remained stable. Notwithstanding that this observation was limited to 1 patient, this finding could suggest that, in some cases, clinical remission may be driven by a de novo differentiation of IgG1+ B cells from the naïve B-cell pool or recruitment of IgG1-switched memory cells.[22] The immunobiological mechanisms underlying the unusual preferential recruitment of IgG1+ instead of IgG4+ MuSK-specific B cells need further investigations.

Measurement of autoantibody affinity was possible in a very limited number of serum samples. In contrast to the successful application of this method for the characterization of the humoral response against SARS-CoV-2,[23] MuSK autoantibody affinity was generally tighter than can be characterized using the technique, thus preventing quantification. This is likely due to a longer (chronic) exposure to the autoantigen and the associated extensive affinity maturation, compared with infectious diseases. Nonetheless, our data show that, in 1 patient not receiving immunotherapy, MuSK antibody affinity increased in parallel with symptom exacerbation while MuSK-IgG and MuSK-IgG4 levels remained stable, suggesting that affinity maturation was a driver of clinical worsening and confirming that affinity maturation is a key step for MuSK antibody pathogenicity, as already demonstrated.[24] Despite being limited to a single observation, our findings support this paradigm and highlight how a multimodal investigational approach, including the quantification of antibody affinity, may provide deeper insights into the complex immunobiology of autoimmune disorders and could lead to the identification of novel serologic biomarkers of clinical outcomes.

This study has limitations inherent to its retrospective design and the relatively small sample size, the latter factor being explained by the rarity of the disorder. Moreover, this study was not powered for (nor aimed to) a cross-sectional analysis for the identification of the demographic and clinical factors

associated with positive outcomes, which awaits to be investigated in the future. Nonetheless, through the comprehensive evaluation of MuSK antibody levels and their IgG subclasses, we could confirm that measurement of total MuSK-IgG and MuSK-IgG4 levels represents a valuable biomarker of disease severity and outcomes and can guide treatment choices. Upon validation in prospective studies, a reduction of MuSK-IgG (or IgG4) could be used as a serologic biomarker to support immunotherapy tapering or suspension. In addition, our data suggest a potential contribution of MuSK-IgG2 antibodies to the disease pathogenesis that deserves further investigations. Finally, our results support the use of flow cytometry–assessed live CBA for the quantification of MuSK-IgG and its subclasses. Multicenter prospective studies are needed to confirm our results and identify reliable, predictive biomarkers that can support a personalized treatment approach in this disorder.

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