PLOS ONE

RESEARCH ARTICLE

Myasthenia gravis complement activity is independent of autoantibody titer and disease severity

Miriam L. Fichtner[1,2], Michelle D. Hoarty[3], Douangsone D. Vadysirisack[3], Bailey Munro-Sheldon[1], Richard J. Nowak[1], Kevin C. O'Connor[1,2]*

1 Department of Neurology, Yale School of Medicine, New Haven, Connecticut, United States of America, **2** Department of Immunobiology, Yale School of Medicine, New Haven, Connecticut, United States of America, **3** UCB Pharma, Cambridge, Massachusetts, United States of America

* kevin.oconnor@yale.edu

Citation: Fichtner ML, Hoarty MD, Vadysirisack DD, Munro-Sheldon B, Nowak RJ, O'Connor KC (2022) Myasthenia gravis complement activity is independent of autoantibody titer and disease severity. PLoS ONE 17(3): e0264489. https://doi.org/10.1371/journal.pone.0264489

Editor: Güher Saruhan-Direskeneli, Istanbul Universitesi Istanbul Medical Faculty, TURKEY

Received: June 9, 2021

Accepted: February 12, 2022

Published: March 15, 2022

Data Availability Statement: This information will only be available after acceptance.

Funding: This project was supported by grants from Ra Pharmaceuticals, now a part of UCB Pharma awarded to KCO and RJN, and by a High Impact Clinical Research and Scientific Pilot Project award from the Myasthenia Gravis Foundation of American (MGFA). Additionally, this work was also supported by the National Institute of Allergy and Infectious Diseases of the NIH (Grant Nos. R01-AI114780 and R21 AI164590) awarded to KCO;

Abstract

Acetylcholine receptor (AChR) autoantibodies, found in patients with autoimmune myasthenia gravis (MG), can directly contribute to disease pathology through activation of the classical complement pathway. Activation of the complement pathway in autoimmune diseases can lead to a secondary complement deficiency resulting in reduced complement activity, due to consumption, during episodes of disease activity. It is not clear whether complement activity in MG patients associates with measurements of disease activity or the titer of circulating pathogenic AChR autoantibodies. To explore such associations, as a means to identify a candidate biomarker, we measured complement activity in AChR MG samples (N = 51) using a CH50 hemolysis assay, then tested associations between these values and both clinical status and AChR autoantibody titer. The majority of the study subjects (88.2%) had complement activity within the range defined by healthy controls, while six patients (11.8%) showed reduced activity. No significant association between complement activity and disease status or AChR autoantibody titer was observed.

Introduction

The most common subtype of autoimmune myasthenia gravis (MG) is characterized by pathogenic autoantibodies targeting the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction [1]. These autoantibodies directly contribute to disease pathology primarily, though not exclusively, through activation of complement. The complement system is part of the innate immune system and an important link between the innate and adaptive immune response [2, 3]. Three different activation pathways and over thirty different proteins are associated with the complement system [4]. The three different complement activation pathways, namely the classical, alternative and lectin pathway, differ in their initial steps, but all converge at the C3 activation step [5]. The classical pathway is activated when C1q binds to antibody–antigen complexes [6], the alternative pathway is the result of spontaneous hydrolysis of C3 which can lead to rapid complement activation on foreign cell surfaces [7] and the lectin

and by a Neuromuscular Disease Research program award from the Muscular Dystrophy Association (MDA) (Grant No. MDA575198) awarded to KCO. MLF received the SPIN award from Grifols and has further been supported through a DFG Research fellowship (FI 2471/1-1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have read the journal's policy and have the following competing interests: KCO has received research support from Ra Pharma, now a part of UCB Pharma. DDV and MDH are paid employees of UCB Pharma. RJN has received research support from UCB Pharma and served as consultant/advisor for UCB Pharma. The study did not receive funding via any of the authors' salaries. These competing interests do not alter our adherence to PLOS ONE policies on sharing data and materials. There are no patents, products in development or marketed products associated with this research to declare.

pathway is activated by the mannose binding lectin (MBL) complex recognizing carbohydrates like mannose on the cell surface of pathogens [8].

Deficiencies of the complement system can be categorized as either hereditary or secondary [9, 10]. The prevalence of both deficiencies can be increased in autoimmune disorders in comparison to the general population [10–12]. Secondary deficiencies are the result of increased complement consumption and subsequent reduced complement activity in either an acute process like septic shock [13] or in autoantibody-mediated autoimmune diseases that involve immune-complexes [10]. Several autoimmune diseases including rheumatoid arthritis [14, 15], ANCA-associated vasculitis [16, 17], systemic lupus erythematosus (SLE) [18, 19] and MG [20, 21] can exhibit reduced complement activity due to its increased consumption during episodes of disease activity. In AChR MG patients the reduced activity can associate with localized autoantibody-mediated complement activation at the neuromuscular junction [22–26].

AChR autoantibodies are useful as a diagnostic biomarker. However, their titer, at single time points, does not correlate well with the disease severity and consequently response to treatment [27–30]. Thus, there is a need for reliable biomarkers in MG to follow the disease course, better inform therapeutic decisions and follow response to therapy. It is not clear whether complement activity in MG patients associates with measurements of disease activity or the circulating levels of pathogenic autoantibodies. To explore a candidate MG biomarker, we measured complement activity and investigated associations with disease burden and AChR autoantibody titers in AChR MG patients and controls.

Materials and methods

Patients, controls, and sample handling

This study was approved by the Human Investigation Committee at the Yale School of Medicine (clinicaltrials.gov || NCT03792659). Informed written consent was obtained from all patients. Peripheral blood was collected from AChR MG patients and healthy controls (HC). All AChR MG patients met definitive diagnostic criteria for MG, including positive serology for AChR autoantibodies. We included 40 different unique patients and longitudinally collected samples from some patients resulting in a total of 51 AChR MG samples (mean age: 59.3 +/- 18.8 yrs) and 20 unique HC samples (34.4 +/- 13.3 yrs) (Table 1). The treatment status of the MG patient cohort (N = 51) was heterogeneous: immunotherapy naïve without any prior treatment (patients that never received treatment for MG or any other autoimmune disease; N = 22), no current therapy, but with prior treatment (specifically, patients that were, at the time of collection and three months prior, not receiving any current treatment for MG; N = 4), cholinesterase inhibitor (N = 10), corticosteroids (N = 10), both corticosteroids and azathioprine (N = 1), both corticosteroids and cholinesterase inhibitor (N = 2), IVIg (N = 1), or PLEx (N = 1). Several studies indicate an effect of IVIg on complement activity [31, 32], but this is effect reverts to normal values around 2 weeks after treatment [32]. Therefore, serum samples were only included if the patient did not receive IVIg within the last two weeks. Patient and healthy control sera were processed within 1 h after collection. Clotting was allowed to proceed for 30 min in the collection tune (BD Vacutainer®, Serum Blood Collection tube, catalog # 366431), then the tube was centrifuged at 2000 g for 15 min at 4˚C. Serum was aliquoted and stored at -80˚C before use. The sera were thawed and kept on ice on the day of the experiment. The burden of disease at time of collection was measured by MG Composite (MGC) score. The MGC score is used to measure the clinical status of MG patients by assessing the extent of muscle weakness [33].

Table 1. Clinical characteristics and demographics of MG patients.

Characteristics	Number
Samples	51
Patients	40
Male / female	26 / 14
Age, yrs (with std. dev.)	59 (+/- 18.8)
Thymectomy	17
Thymoma	4
Thymus hyperplasia	7
Early Onset MG	17
Late Onset MG	34
MGFA classification	
I	21
II	12
III	7
IV	1
V	2
No current symptoms	8
Treated	25
Prednisone	10
Pyridostigmine	10
Prednisone + Azathioprine	1
Prednisone + Pyridostigmine	2
PLEx	1
IVIg	1
Untreated	26
Immunotherapy naïve	22
No current therapy	4

Abbreviations: yrs, years; MGFA, Myasthenia Gravis Foundation of America; PLEx, plasma exchange; IVIg, intravenous immunoglobulin. No. or mean +/- SD (range) are shown.

https://doi.org/10.1371/journal.pone.0264489.t001

CH50 hemolysis assay

The CH50 hemolysis assay measures the complement activity of the antibody-dependent classical complement pathway by measuring the capability of the serum complement to lyse antibody-sensitized sheep red blood cells (RBC) [34]. We used a previously described established assay for CH50 determination [35–37]. In short, human serum is titrated to obtain the fraction of serum which causes lysis of 50% of the RBCs as measured by the hemoglobin released into the supernatant. Antibody-sensitized sheep erythrocytes (Complement Tech, Tyler TX) were centrifuged for 3 min at 1000 g. The supernatant was removed and replaced with an equal volume of fresh gelatin veronal buffered saline containing 0.15 mM calcium chloride and 0.5 mM magnesium chloride (GVB++; Complement Tech, Tyler Tx). The sheep erythrocytes were at a concentration of 5 x 10^8 cells per ml. Human sera samples were diluted in 10 serial dilutions (2-fold each) in GVB++. Sheep erythrocytes (100 μl), 50 μl of GVB++ and 50 μl of the serum sample were added into 96-well tissue culture treated plates (USA Scientific) and mixed by pipetting up and down three times. The plate was incubated at 37˚C for 1 h. After incubation the plate was centrifuged at 1000 g for 3 min and 100 μl of the supernatant was transferred to a new plate without disturbing the pellet. The absorbance of the hemoglobin released into the

supernatant was read at 412 nm with the Infinite® 200 PRO (Tecan Life Sciences, Switzerland). Assay controls consisted of GVB++ buffer only as the background of the plate, sheep erythrocytes with GVB++ as the background of lysis, and sheep erythrocytes lysed with Triton X100 for total lysis. Additionally, we included the same sample (standard) for inter-assay standardization. Only assay results with a range of values +/- 20% within the standard sample were included in the final analysis.

Statistics

Statistics were calculated with Prism Software (GraphPad; version 8.0). Descriptive statistics were used to evaluate the mean of age between the AChR MG and HC cohort and to set the CH50 assay cut-off for low complement activity (mean +/- 2 SD). Spearman correlation was used to assess the correlation of AChR autoantibody titers and disease burden (measured by MGC score) to CH50 assay values. Bonferroni correction was used to adjust for multiple tests.

Results

AChR MG patients (N = 51, mean age: 59.3 +/- 18.8 yrs; (Table 1)) and healthy controls (HC; N = 20, mean age: 34.4 +/- 13.3 yrs) were tested for complement activity by hemolytic CH50 assay. The majority of AChR MG samples (88.2%) had complement activity within the range defined by the HCs (Fig 1A). Six AChR MG samples (11.8%) had reduced complement activity (Fig 1A; Table 2). Three out of the six samples with reduced complement activity were immunotherapy naïve at the time of collection (Table 2).

The largest subcohort within our samples consisted of immunotherapy naïve samples (43.1%). We first compared the complement activity between immunotherapy naïve and non-immunotherapy naïve samples and found no significant difference between these two groups (Fig 1B). Recent studies indicate that complement activity increases with age; one such study found differences when comparing the values of participants over 60 years of age to younger participants [38]. This age-dependent increased complement activity could possibly conceal reduced levels. Our patient cohort included patients (57%) at the age of 60 years and older. However, we found no significant difference of complement activation between MG patients 60 years of age and older at time of collection to MG patients younger than 60 years of age (Fig 1C). Serial samples of one patient with reduced complement activity were tested to investigate how the activity changed over time. The complement activity and disease burden (MGC score) of this patient normalized over the time course of 21 months (Fig 1D; Table 3). Next, we investigated whether complement activity within our cohort of 51 patients associates with MG disease burden or the circulating levels of AChR autoantibodies. No association (ns; $p = 0.8$) between disease burden (MGC score) and complement activity was observed (Fig 1E). Similarly, no significant (ns; $p = 0.5$) correlation between complement activity and AChR autoantibody titer was observed (Fig 1F). We further analyzed the correlation of complement activity to MGC score and AChR autoantibody titer by comparing subcohorts defined by complement activity, age of disease onset (early onset MG (EOMG) for onset before the age of 50 years; late onset MG (LOMG) for onset on or after the age of 50 years), treatment status and thymoma and found no significant correlations (Fig 2A–2H).

Discussion

In this study we measured the total complement activity by the CH50 hemolysis assay. The findings suggest that complement activity in most AChR MG patients is within the range defined by HC. Decreased complement activity was observed in a small subgroup. While secondary deficiencies of complement activity can arise during episodes of autoimmune disease

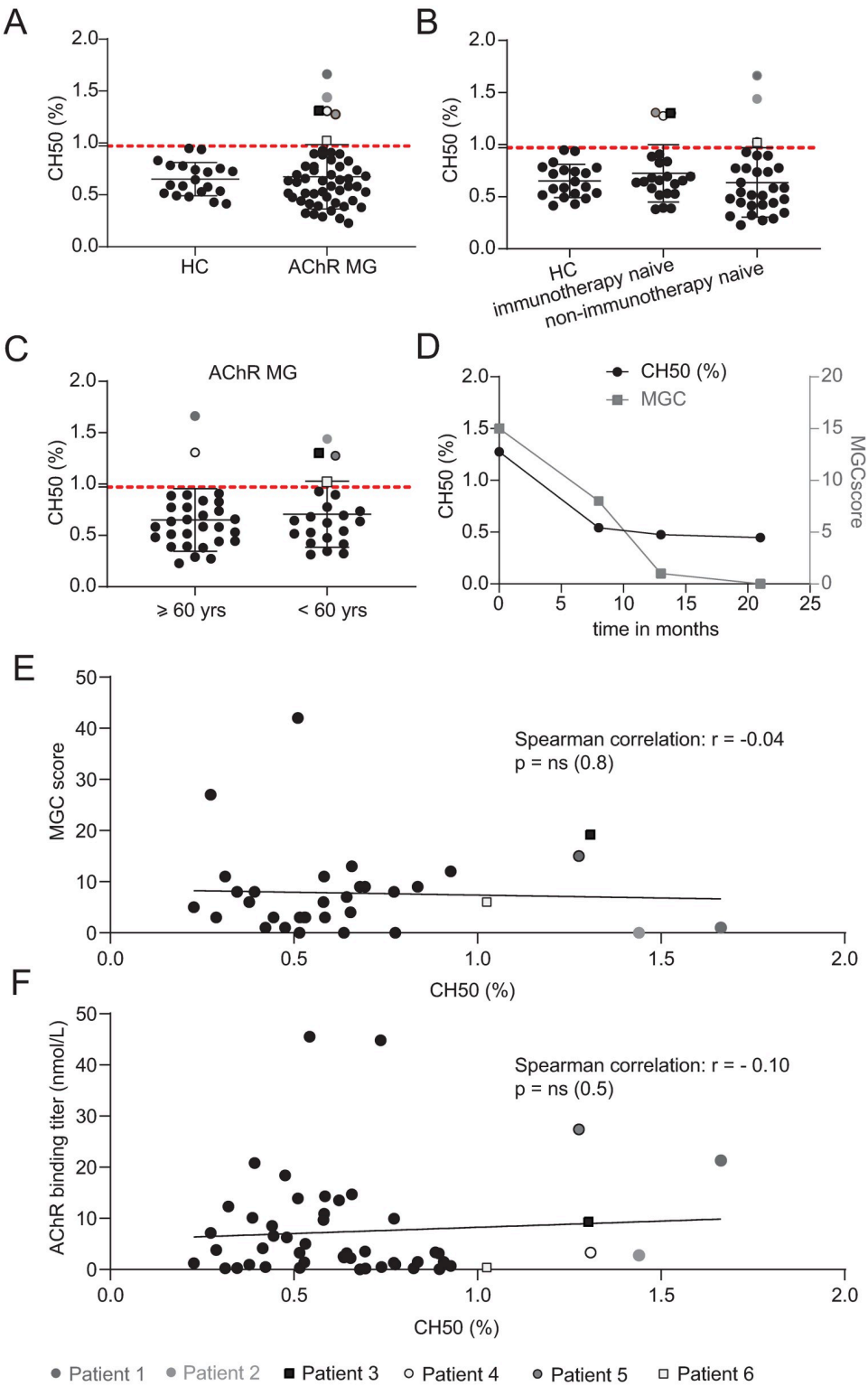

Fig 1. Complement activity in AChR MG does not associate with clinical status or autoantibody titer. Complement activity in the serum of AChR MG patients was measured by CH50 hemolysis assay. The assay measures the activation of the classical complement pathway by testing the ability of the complement components of sera samples to lyse antibody-sensitized sheep erythrocytes. The CH50 values are given as the percentage (%) of serum needed to lyse 50% of sheep erythrocytes. (A) AChR MG patients (N = 51) and healthy controls (N = 20) were

measured by CH50 hemolysis assay. (B) Comparison of the complement activity between immunotherapy naïve (N = 22) vs non-immunotherapy naïve (N = 29) AChR MG patients. (C) Comparison of the complement activity between AChR MG patients 60 years of age and older (N = 29) vs AChR MG patients younger than 60 years of age (N = 22). (D) Serial samples of patient 5 (Table 3) were measured by CH50 hemolysis assay and compared to the corresponding disease burden (MGC score) at the time of each collection. The X axis shows the time in months since the first sample collection, the left Y axis shows the CH50 values (%) and the right Y axis shows the Myasthenia Gravis Composite (MGC) score. (E) Correlation of complement activity with the MGC score. The MGC score values were available for 33 of the 51 patients. (F) Correlation of complement activity with the AChR antibody titer (N = 51). The linear regression is shown with Spearman correlation values. Patients 1–6 (Table 2) are individually illustrated (see legend) with their corresponding MGC score (E) or antibody titer (F) if values were available. Values higher than the mean + 2SD of the HC controls (indicated by the horizontal dotted line) were considered reduced (A-C).

https://doi.org/10.1371/journal.pone.0264489.g001

Table 2. Characteristics of six AChR MG patients with reduced complement activity.

Subject ID	Age at TOC, Sex	EOMG/LOMG	Antibody titer	MGFA class at TOC	Treatment at TOC	Immunotherapy naïve at TOC	Thymectomy
Patient 1	72, M	LOMG	21.3	I	Mestinon 180 mg/d	No	-
Patient 2	36, F	EOMG	2.79	0	-	No	4
Patient 3	46, F	EOMG	9.21	IIIA	-	Yes	1.5
Patient 4	72, M	LOMG	3.31	I	-	Yes	-
Patient 5	32, M	EOMG	27.4	IIIA	-	Yes	-
Patient 6	57, M	LOMG	0.43	I	Mestinon 540 mg/d	No	-

Antibody titer was measured at the Mayo Clinic Laboratory; the unit is nmol/L, the cut off for negativity is $\leq$ 0.02 nmol/L. The values for thymectomy represent the time in years since thymectomy. MGFA class = Myasthenia Gravis Foundation of America classification; TOC = time of collection; EOMG = early-onset myasthenia gravis; LOMG = late-onset MG.

https://doi.org/10.1371/journal.pone.0264489.t002

activity, we observed no association between complement activity and AChR autoantibody titer or between complement activity and disease activity.

We chose to focus on the CH50 hemolysis assay because it is a functional assay that provides a quantitative measurement of the classical pathway overall activity. While the CH50 hemolysis assay is widely used and highly reliable, there are limitations that must be recognized. One limitation of the CH50 hemolysis assay is that it is susceptible to acute inflammatory processes which lead to increased complement activity possibly concealing reduced activity [39]. The patients in our study cohort did not have any apparent acute non-MG related inflammation. Another limitation is that while the assay measures the functionality of the classical complement pathway, it cannot be used to identify altered levels of specific proteins within that pathway. Circulating levels of C3 and C4 have been investigated in MG with divergent results. Their levels were found to be the same as those found in HC [40, 41] or

Table 3. Patient characteristics during longitudinal sample collection.

Sample Collection (months)	Antibody titer	MGFA class, and MGC score at TOC	Treatment at TOC
0	27.4	IIIA; 15	-
8	45.5	IIB; 8	Pred 20 mg/d
13	18.4	I; 1	Pred 10 mg/d, IVIg
21	10.3	0; 0	IVIg

The time point 0 is normalized to indicate the first sample (Patient 5 (Table 1)) in the series. The values for timepoints within the serial sample represent the time in months since the first sample. Antibody titer was measured at the Mayo Clinic Laboratory; the unit is nmol/L, the cut off for negativity is $\leq$ 0.02 nmol/L. TOC = time of collection; MGFA class = Myasthenia Gravis Foundation of America classification; MGC score = MG Composite score; Pred = prednisone.

https://doi.org/10.1371/journal.pone.0264489.t003

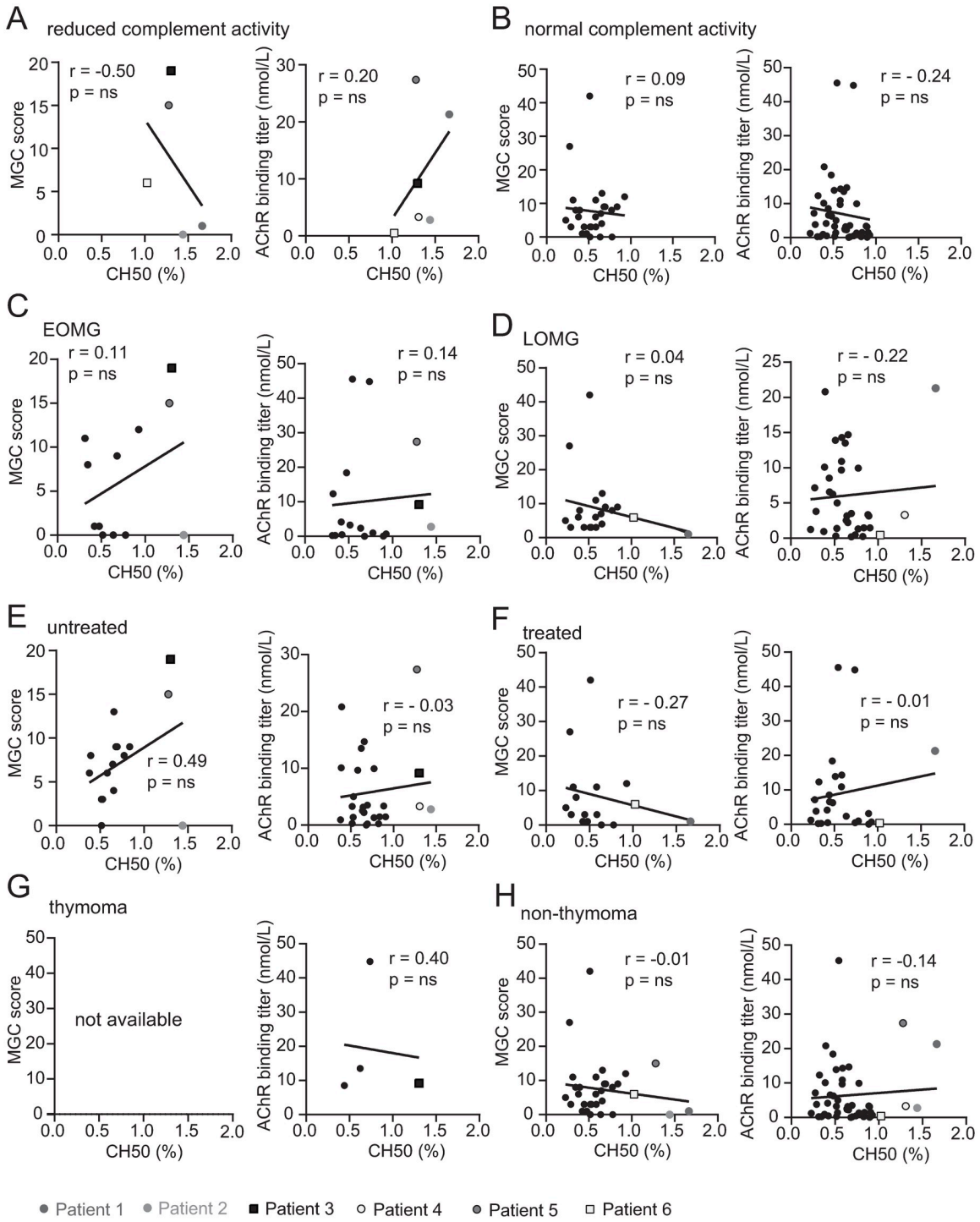

Fig 2. Correlation between complement activity and MG patient subcohort demographics. Correlation tests between complement activity to clinical status and autoantibody titer in different MG patient subcohorts defined by complement activity, age of disease onset, treatment status, and thymoma. (A-H) Correlation of complement activity with MGC score (first and third columns) and AChR antibody titer (second and fourth columns). The subcohorts are defined by complement activity (A and B), age of disease onset (early onset MG (EOMG; C); late onset MG (LOMG; D)), treatment status (E and F) and thymoma status (G and H). Limited specimens (n = 1) in the thymoma category with matching MGC scores prohibited correlative analysis (G left panel). The linear regression is shown with Spearman correlation values. The Bonferroni correction was used to adjust for multiple tests. Patient 1–6 (Table 2) are individually illustrated (see legend) for each panel.

https://doi.org/10.1371/journal.pone.0264489.g002

conversely, the levels of C3 and C4 were reduced in MG patients [42, 43]. Additionally, increased levels of soluble C5b-9 were found in MG patients in comparison to HCs [41] and C5a levels showed a positive correlation with disease severity [40]. While we identified six patients with diminished complement activity, determining which components were reduced was outside of the scope of the study.

Eculizumab, a therapeutic complement inhibitor, was demonstrated to be effective in a phase 3 trial with subsequent approval for its use in treating AChR receptor antibody-positive MG [44, 45]. Clinical response to this treatment can be heterogeneous; many patients respond well or show a delayed response while, others respond poorly. The mechanisms underlying the variable response are not known. However, there is a need for further understanding so that responses can be better predicted. The assays that are routinely used to diagnose MG, measure the capability of the polyclonal serum derived autoantibodies to bind to the AChR, but not their pathogenic properties. Three major pathogenic mechanisms have been identified for AChR autoantibodies. The first describes AChR autoantibodies blocking the access of acetylcholine to the AChR and thus hindering the neuromuscular signal transduction [46]. The second, termed antigen modulation, describes AChR receptor crosslinking followed by receptor internalization resulting in a reduced number of available receptors [47]. The third involves activation of the classical complement pathway by AChR autoantibodies [22]. There may also be AChR autoantibodies that bind the receptor in the clinical diagnostic assay but have no pathogenic capacity. We propose that the discordance between titer and disease severity and between titer and treatment response may be due to the limitations of diagnostic assays. Specifically, because they measure only binding, they are wholly unable to discriminate between detection of AChR autoantibodies and their pathogenic mechanism or if they are nonpathogenic. Currently, there is no established approach for measuring the complement activating properties of AChR autoantibodies. Given that complement activity in most MG patients is normal and lacks an association with disease activity, such a tool would provide valuable biomarker data for identifying patients expected to respond to complement inhibitor-based treatments.

Supporting information

S1 Dataset.
(PDF)

Acknowledgments

The authors thank Karen Boss for providing editorial assistance.

Author Contributions

Conceptualization: Miriam L. Fichtner, Richard J. Nowak, Kevin C. O'Connor.

Data curation: Miriam L. Fichtner, Bailey Munro-Sheldon.

Formal analysis: Miriam L. Fichtner, Michelle D. Hoarty.

Funding acquisition: Richard J. Nowak, Kevin C. O'Connor.

Investigation: Miriam L. Fichtner, Richard J. Nowak, Kevin C. O'Connor.

Methodology: Miriam L. Fichtner, Michelle D. Hoarty, Douangsone D. Vadysirisack.

Resources: Richard J. Nowak, Kevin C. O'Connor.

Supervision: Kevin C. O'Connor.

Validation: Miriam L. Fichtner, Michelle D. Hoarty, Douangsone D. Vadysirisack.

Visualization: Miriam L. Fichtner.

Writing – original draft: Miriam L. Fichtner, Kevin C. O'Connor.

Writing – review & editing: Miriam L. Fichtner, Michelle D. Hoarty, Douangsone D. Vadysirisack, Richard J. Nowak, Kevin C. O'Connor.

References

1. Vincent A. Unravelling the pathogenesis of myasthenia gravis. Nature reviews Immunology. 2002; 2 (10):797–804. https://doi.org/10.1038/nri916 PMID: 12360217.
2. Janeway CA Jr T P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. New York: Garland Science; 2001.
3. Dunkelberger JR, Song W-C. Complement and its role in innate and adaptive immune responses. Cell Research. 2010; 20(1):34–50. https://doi.org/10.1038/cr.2009.139 PMID: 20010915
4. Walport MJ. Complement. First of two parts. N Engl J Med. 2001; 344(14):1058–66. Epub 2001/04/05. https://doi.org/10.1056/NEJM200104053441406 PMID: 11287977.
5. Sarma JV, Ward PA. The complement system. Cell and tissue research. 2011; 343(1):227–35. Epub 2010/09/15. https://doi.org/10.1007/s00441-010-1034-0 PMID: 20838815
6. Mortensen SA, Sander B, Jensen RK, Pedersen JS, Golas MM, Jensenius JC, et al. Structure and activation of C1, the complex initiating the classical pathway of the complement cascade. Proceedings of the National Academy of Sciences. 2017; 114(5):986. https://doi.org/10.1073/pnas.1616998114 PMID: 28104818
7. Thurman JM, Holers VM. The Central Role of the Alternative Complement Pathway in Human Disease. The Journal of Immunology. 2006; 176(3):1305. https://doi.org/10.4049/jimmunol.176.3.1305 PMID: 16424154
8. Fujita T. Evolution of the lectin-complement pathway and its role in innate immunity. Nature reviews Immunology. 2002; 2(5):346–53. Epub 2002/05/30. https://doi.org/10.1038/nri800 PMID: 12033740.
9. Nusinow SR, Zuraw BL, Curd JG. The hereditary and acquired deficiencies of complement. Med Clin North Am. 1985; 69(3):487–504. Epub 1985/05/01. https://doi.org/10.1016/s0025-7125(16)31028-8 PMID: 3892188.
10. Wen L, Atkinson JP, Giclas PC. Clinical and laboratory evaluation of complement deficiency. J Allergy Clin Immunol. 2004; 113(4):585–93; quiz 94. Epub 2004/04/22. https://doi.org/10.1016/j.jaci.2004.02.003 PMID: 15100659.
11. Johnson CA, Densen P, Wetsel RA, Cole FS, Goeken NE, Colten HR. Molecular Heterogeneity of C2 Deficiency. New England Journal of Medicine. 1992; 326(13):871–4. https://doi.org/10.1056/NEJM199203263261306 PMID: 1542325.
12. Atkinson JP. Complement deficiency: predisposing factor to autoimmune syndromes. Clin Exp Rheumatol. 1989; 7 Suppl 3:S95–101. Epub 1989/09/01. PMID: 2691164.
13. León C, Rodrigo MJ, Tomasa A, Gallart MT, Latorre FJ, Rius J, et al. Complement activation in septic shock due to gram-negative and gram-positive bacteria. Crit Care Med. 1982; 10(5):308–10. Epub 1982/05/01. PMID: 6918253.
14. Tomooka K. [Serum complement levels in patients with rheumatoid arthritis and vasculitis]. Fukuoka Igaku Zasshi. 1989; 80(10):456–66. Epub 1989/10/01. PMID: 2613159.
15. Rynes RI, Ruddy S, Spragg J, Sydney Stillman J, Frank Austen K. Intraarticular activation of the complement system in patients with juvenile rheumatoid arthritis. Arthritis & Rheumatism. 1976; 19(2):161–8. https://doi.org/10.1002/art.1780190206 PMID: 1259792
16. Fukui S, Iwamoto N, Umeda M, Nishino A, Nakashima Y, Koga T, et al. Antineutrophilic cytoplasmic antibody-associated vasculitis with hypocomplementemia has a higher incidence of serious organ damage and a poor prognosis. Medicine (Baltimore). 2016; 95(37):e4871–e. https://doi.org/10.1097/MD.0000000000004871 PMID: 27631255.
17. García L, Pena CE, Maldonado RÁ, Costi C, Mamberti M, Martins E, et al. Increased renal damage in hypocomplementemic patients with ANCA-associated vasculitis: retrospective cohort study. Clinical Rheumatology. 2019; 38(10):2819–24. https://doi.org/10.1007/s10067-019-04636-9 PMID: 31222573

18. Pickering MC, Walport MJ. Links between complement abnormalities and systemic lupus erythematosus. Rheumatology. 2000; 39(2):133–41. https://doi.org/10.1093/rheumatology/39.2.133 PMID: 10725062

19. Agnello V. Lupus diseases associated with hereditary and acquired deficiencies of complement. Springer Seminars in Immunopathology. 1986; 9(2):161–78. https://doi.org/10.1007/BF02099020 PMID: 3544278

20. Nastuk WLO, K. E.; and Plescia O.J. Reduction in Serum Complement Concentration in Myasthenia Gravis Federation Proceedings. 1956; 15:135–6.

21. Behan WM, Behan PO. Immune complexes in myasthenia gravis. J Neurol Neurosurg Psychiatry. 1979; 42(7):595–9. https://doi.org/10.1136/jnnp.42.7.595 PMID: 479898.

22. Engel AG, Lambert EH, Howard FM. Immune complexes (IgG and C3) at the motor end-plate in myasthenia gravis: ultrastructural and light microscopic localization and electrophysiologic correlations. Mayo Clin Proc. 1977; 52(5):267–80. Epub 1977/05/01. PMID: 870771

23. Engel AG, Sakakibara H, Sahashi K, Lindstrom JM, Lambert EH, Lennon VA. Passively transferred experimental autoimmune myasthenia gravis. Sequential and quantitative study of the motor end-plate fine structure and ultrastructural localization of immune complexes (IgG and C3), and of the acetylcholine receptor. Neurology. 1979; 29(2):179–88. Epub 1979/02/01. https://doi.org/10.1212/wnl.29.2.179 PMID: 571062.

24. Nakano S, Engel AG. Myasthenia gravis: quantitative immunocytochemical analysis of inflammatory cells and detection of complement membrane attack complex at the end-plate in 30 patients. Neurology. 1993; 43(6):1167–72. https://doi.org/10.1212/wnl.43.6.1167 PMID: 8170563.

25. Sahashi K, Engel AG, Lambert EH, Howard FM, Jr. Ultrastructural localization of the terminal and lytic ninth complement component (C9) at the motor end-plate in myasthenia gravis. J Neuropathol Exp Neurol. 1980; 39(2):160–72. Epub 1980/03/01. https://doi.org/10.1097/00005072-198003000-00005 PMID: 7373347.

26. Strauss AJL, Seegal BC, Hsu KC, Burkholder PM, Nastuk WL, Osserman KE. Immunofluorescence Demonstration of a Muscle Binding, Complement-Fixing Serum Globulin Fraction in Myasthenia Gravis. Proceedings of the Society for Experimental Biology and Medicine. 1960; 105(1):184–91. https://doi.org/10.3181/00379727-105-26051

27. Lefvert AK, Bergström K, Matell G, Osterman PO, Pirskanen R. Determination of acetylcholine receptor antibody in myasthenia gravis: clinical usefulness and pathogenetic implications. Journal of neurology, neurosurgery, and psychiatry. 1978; 41(5):394–403. Epub 1978/05/01. https://doi.org/10.1136/jnnp.41.5.394 PMID: 207825

28. Lindstrom JM, Seybold ME, Lennon VA, Whittingham S, Duane DD. Antibody to acetylcholine receptor in myasthenia gravis. Prevalence, clinical correlates, and diagnostic value. Neurology. 1976; 26 (11):1054–9. Epub 1976/11/01. https://doi.org/10.1212/wnl.26.11.1054 PMID: 988512.

29. Tindall RS. Humoral immunity in myasthenia gravis: clinical correlations of anti-receptor antibody avidity and titer. Annals of the New York Academy of Sciences. 1981; 377:316–31. Epub 1981/01/01. https://doi.org/10.1111/j.1749-6632.1981.tb33741.x PMID: 6803647.

30. Vincent A, Newsom Davis J. Anti-acetylcholine receptor antibodies. Journal of neurology, neurosurgery, and psychiatry. 1980; 43(7):590–600. Epub 1980/07/01. https://doi.org/10.1136/jnnp.43.7.590 PMID: 7400823

31. Mollnes TE, Andreassen IH, Høgåsen K, Hack CE, Harboe M. Effect of whole and fractionated intravenous immunoglobulin on complement in vitro. Mol Immunol. 1997; 34(10):719–29. Epub 1997/07/01. https://doi.org/10.1016/s0161-5890(97)00091-6 PMID: 9430199.

32. Machimoto T, Guerra G, Burke G, Fricker FJ, Colona J, Ruiz P, et al. Effect of IVIG administration on complement activation and HLA antibody levels. Transpl Int. 2010; 23(10):1015–22. Epub 2010/04/24. https://doi.org/10.1111/j.1432-2277.2010.01088.x PMID: 20412537.

33. Burns TM, Conaway MR, Cutter GR, Sanders DB, Muscle Study G. Construction of an efficient evaluative instrument for myasthenia gravis: the MG composite. Muscle & nerve. 2008; 38(6):1553–62. Epub 2008/11/20. https://doi.org/10.1002/mus.21185 PMID: 19016543.

34. Costabile M. Measuring the 50% haemolytic complement (CH50) activity of serum. Journal of visualized experiments: JoVE. 2010;(37):1923. https://doi.org/10.3791/1923 PMID: 20351687.

35. Howard JF, Kaminski HJ, Nowak RJ, Wolfe GI, Benatar MG, Ricardo A, et al. RA101495, A Subcutaneously Administered Peptide Inhibitor of Complement Component 5 (C5) for the Treatment of Generalized Myasthenia Gravis (gMG): Phase 1 Results and Phase 2 Design (S31.006). Neurology. 2018; 90 (15 Supplement):S31.006.

36. Ricardo A, Hoarty M, Ma Z, Rajagopal V, Seyb K, Tang G-Q, et al. RA101495 abolishes residual complement activity observed in the presence of eculizumab. Molecular immunology. 2017; 89:189. https://doi.org/https%3A//doi.org/10.1016/j.molimm.2017.06.186

37. Howard JF Jr., Nowak RJ, Wolfe GI, Freimer ML, Vu TH, Hinton JL, et al. Clinical Effects of the Self-administered Subcutaneous Complement Inhibitor Zilucoplan in Patients With Moderate to Severe Generalized Myasthenia Gravis: Results of a Phase 2 Randomized, Double-Blind, Placebo-Controlled, Multicenter Clinical Trial. JAMA Neurol. 2020; 77(5):582–92. https://doi.org/10.1001/jamaneurol.2019.5125 PMID: 32065623

38. Gaya da Costa M, Poppelaars F, van Kooten C, Mollnes TE, Tedesco F, Würzner R, et al. Age and Sex-Associated Changes of Complement Activity and Complement Levels in a Healthy Caucasian Population. Frontiers in immunology. 2018; 9:2664-. https://doi.org/10.3389/fimmu.2018.02664 PMID: 30515158.

39. Glovsky MM, Ward PA, Johnson KJ. Complement determinations in human disease. Annals of allergy, asthma & immunology: official publication of the American College of Allergy, Asthma, & Immunology. 2004; 93(6):513–22; quiz 23–5, 605. Epub 2004/12/22. https://doi.org/10.1016/S1081-1206(10)61257-4 PMID: 15609759.

40. Aguirre F, Manin A, Fernandez VC, Justo ME, Leoni J, Paz ML, et al. C3, C5a and anti-acetylcholine receptor antibody as severity biomarkers in myasthenia gravis. Therapeutic advances in neurological disorders. 2020; 13:1756286420935697. Epub 2020/08/28. https://doi.org/10.1177/1756286420935697 PMID: 32843900

41. Ozawa Y, Uzawa A, Yasuda M, Kojima Y, Oda F, Himuro K, et al. Changes in serum complements and their regulators in generalized myasthenia gravis. European journal of neurology. 2021; 28(1):314–22. Epub 2020/09/06. https://doi.org/10.1111/ene.14500 PMID: 32889770.

42. Romi F, Kristoffersen EK, Aarli JA, Gilhus NE. The role of complement in myasthenia gravis: serological evidence of complement consumption in vivo. Journal of neuroimmunology. 2005; 158(1–2):191–4. Epub 2004/12/14. https://doi.org/10.1016/j.jneuroim.2004.08.002 PMID: 15589053.

43. Liu A, Lin H, Liu Y, Cao X, Wang X, Li Z. Correlation of C3 level with severity of generalized myasthenia gravis. Muscle & nerve. 2009; 40(5):801–8. Epub 2009/08/12. https://doi.org/10.1002/mus.21398 PMID: 19670317.

44. Howard JF Jr., Utsugisawa K, Benatar M, Murai H, Barohn RJ, Illa I, et al. Safety and efficacy of eculizumab in anti-acetylcholine receptor antibody-positive refractory generalised myasthenia gravis (REGAIN): a phase 3, randomised, double-blind, placebo-controlled, multicentre study. Lancet Neurol. 2017; 16(12):976–86. Epub 2017/10/27. https://doi.org/10.1016/S1474-4422(17)30369-1 PMID: 29066163.

45. Muppidi S, Utsugisawa K, Benatar M, Murai H, Barohn RJ, Illa I, et al. Long-term safety and efficacy of eculizumab in generalized myasthenia gravis. Muscle & nerve. 2019; 60(1):14–24. Epub 2019/02/16. https://doi.org/10.1002/mus.26447 PMID: 30767274

46. Almon RR, Andrew CG, Appel SH. Serum globulin in myasthenia gravis: inhibition of alpha-bungarotoxin binding to acetylcholine receptors. Science (New York, NY). 1974; 186(4158):55–7. Epub 1974/10/04. https://doi.org/10.1126/science.186.4158.55 PMID: 4421998.

47. Drachman DB, Angus CW, Adams RN, Michelson JD, Hoffman GJ. Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation. N Engl J Med. 1978; 298(20):1116–22. Epub 1978/05/18. https://doi.org/10.1056/NEJM197805182982004 PMID: 643030.