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Short Communication

Elevated C1s/C1-INH in serum and plasma of myasthenia gravis patients

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ABSTRACT

Myasthenia Gravis (MG) is an autoimmune neuromuscular disorder where acetylcholine receptor (AChR) antibodies induce membrane attack complex formation at the muscle membrane. The C1-inhibitor (C1-INH) regulates the classical pathway and is a promising marker in other autoimmune disorders. Treatment options for AChR antibody MG include complement inhibitors; nevertheless, the early pathway activation in MG remains unclear. Serum and plasma C1s-C1-INH levels were higher in MG patients than in matched healthy controls, supporting early classical pathway activation in most MG patients. These findings allow prospective validation studies of activated C1s as a putative treatment target and potential accompanying biomarker in MG.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease where immunoglobulin G (IgG) antibodies attack acetylcholine receptor (AChR) or AChR clustering associated proteins at the neuromuscular junction (NMJ), causing the failure of neuromuscular transmission and ultimately leading to fluctuating skeletal muscle weakness (Punga and Ruegg, 2012). The IgG1 and IgG3 antibodies specific to AChR are detected in most MG patients and trigger at least three pathogenic mechanisms, including directed blocking of AChR, crossing-linking AChR, and activation of the complement system (Conti-Fine et al., 2006). The activation of the classical complement pathway is essential in AChR antibody seropositive (AChR+) MG pathogenesis, supported by several studies of complement systems in human biopsy and animal models of MG (Chamberlain-Banoub et al., 2006; Engel et al., 1977; Engel et al., 1979; Nastuk et al., 1960; Sahashi et al., 1980; Tuzun and Christadoss, 2013).

The classical pathway of the complement system is activated upon the binding of IgG-antibody-antigen complexes to the C1 complex, which comprises three subunits: recognition subunit C1q and enzymatic subunits C1r and C1s. C1q binding to antibodies attached to antigens initiates the activation of the complement cascade. Once C1q binds to an antibody-antigen complex, it undergoes a conformational change that activates C1r, subsequently then activates C1s. The C1-inhibitor (C1-INH) is the primary regulator of the classical complement pathway, and it functions to control the activation of the C1 complex by binding with C1r and C1s, further regulating the proteolysis-based activation cascade to prevent overreaction of the immune system (Hurler et al., 2022; Zeerleder, 2011). After the binding of C1-INH to C1r and C1s, the C1 complex separates, releasing free C1q along with covalent C1r/C1-INH and C1s/C1-INH complexes. The elevated levels of C1s/C1-INH are detected in diseases such as hereditary angioedema, systemic lupus erythematosus, glomerulonephritis, and rheumatoid arthritis(Auda et al., 1990; Kajdacsi et al., 2020; Nielsen et al., 1995; Waldo and West, 1987), suggesting C1s/C1-INH as a promising marker for monitoring early classical pathway activation. Like other arms of the complement pathway, classical pathway activation produces anaphylatoxins C3a and C5a, amplifying inflammatory responses and forming the membrane attack complex (MAC). The MAC is the effector arm of the complement cascade and induces direct lysis of cells (Lu and Kishore, 2017; Sarma and Ward, 2011). In AChR+ MG, the activation of the classical complement pathway results in the formation of MAC on the neuromuscular junction, focal lysis of the postsynaptic membrane, disruption of postjunctional folds, and ultimately reduction in functional AChRs, compromising neuromuscular transmission (Conti-Fine et al., 2006; Liu et al., 2011; Tuzun and Christadoss, 2013). Terminal complement components have been detected in the sera and plasma of patients with MG, although this detection is not necessarily linked to disease severity (Barohn and Brey, 1993; Romi et al., 2005). However, there is limited data on proximal classical pathway complement components, such as active C1s, in human sera or plasma in patients with MG.

Our objective in this study was to measure the C1s/C1-INH complex levels in the sera and plasma of patients with MG. Based on the expected role of the classical pathway in MG disease pathology, we hypothesized

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that patients with MG would have elevated levels of C1s/C1-INH compared to matched healthy controls. We further aimed to evaluate whether elevated levels of C1s/C1-INH were correlated with other important MG disease characteristics such as clinical severity, concurrent therapies, or disease onset.

2. Materials and methods

2.1. Standard protocol approval and patient consent

This study was approved by the Swedish Ethical Review Authority on human experimentation (ethical permit numbers 2018/446 and 2020–03049), and written informed consent for research was obtained from all MG patients and HCs.

2.2. Myasthenia gravis (MG) patients and controls

Sera were obtained from 73 Swedish MG patients, of which 50 were acetylcholine receptor antibody seropositive (AChR+). Plasma was also obtained from 23 of these MG patients. All patients had an ICD-10 diagnosis of G70.0 with a typical clinical phenotype of objective fatigable skeletal muscle weakness and abnormal electrophysiology by repetitive nerve stimulation (RNS) or single fiber electromyography (SFEMG). Disease severity was assessed through the MG Composite (MGC) score and MG Activities of Daily Living (MG-ADL). Age- and sex-matched healthy control (HC) sera and plasma were collected at Uppsala University Hospital Blood Central. Subgrouping was based on age at onset, into early-onset MG (EOMG; onset 19–50 years) and late-onset MG (LOMG; onset >50 years).

For serum samples, whole blood was collected into Vacutainers tubes without additives and allowed to clot at room temperature for at least 20 min. Samples were then centrifuged at 2200 ×*g* for 10 min. For plasma samples, whole blood was collected into EDTA-treated tubes and then centrifuged at 2200 x*g* for 10 min. Following centrifugation, sera and plasma were transferred to clean polypropylene tubes, then aliquoted and stored at −80 °C until used.

2.3. C1s/C1-INH ELISA testing

Detection of the C1s/C1-INH complex was done using a commercially available C1s/C1-INH human sandwich ELISA kit, and all samples were run according to protocol (HK-399-02, Hycult Biotech, The Netherlands). Controls included C1s-C1INH Complex C1s Enzyme (active C1s) and C1s-depleted human sera, all obtained from Complement Technology, Texas, USA. All samples were assayed in duplicate; the detection range was 1.56–100 ng/mL. The plate was read at 450 nm by CLARIOstar Plus microplate reader (BMG LABTECH).

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc). DÁgostino and Pearson's test was applied for the normality test, and non-parametric data were presented as a median with a 95 % confidence interval. The Wilcoxon-Rank sign test compared non-parametric data paired between MG patients and HCs, and the Mann-Whitney test compared subgroups of MG patients. Correlation analysis was performed using nonparametric Spearman's rank correlation test. Friedman test was used to compare non-parametric data over 4 weeks. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Serum C1s-C1-INH levels are higher in MG than in matched healthy controls

Serum analysis from 73 MG patients (32 men, 59.8 ± 18.2 years; Table 1) and 74 HCs (33 men, 56.3 ± 15.8 years) were included. C1s-C1-INH levels were significantly higher in the MG patient sera (median: 2295 ng/ml; 95 % CI: 1893–2694 ng/ml) than in the HCs (median: 1544 ng/ml; 95 % CI: 1350–1721; $p = 0.0102$; Fig. 1A). There was no difference in C1s-C1-INH levels between patients with and without immunosuppression ($p = 0.4634$; Fig. 1B). MGC scores ranged from 0 to 34 points (median: 4 points; 95 % CI: 3–8 points), and MG-ADL ranged from 0 to 18 (median: 4 points; 95 % CI: 2–5 points). There was no direct correlation between the level of C1s-INH and MGC (R = -0.0187; $p = 0.8754$), MG-ADL (R = -0.01557; $p = 0.8960$) or AChR antibody titer in nM ($R = -0.093$; $p = 0.7647$). Further, since MG can be divided into none-mild symptoms (MG-ADL 0–4 points) and moderate-severe symptoms (MG-ADL of 5 points or more), we compared C1s-C1-INH levels between these clinical severity subgroups. There was no difference in C1s-C1-INH levels between the group of 43 patients with none-mild MG (median: 2338 ng/ml) versus 29 patients with moderate-severe MG (median: 2237 ng/ml; $p = 0.6581$; Fig. 1C). The AChR+ patients ($N = 50$) had comparable C1s-C1-INH levels (median: 2594 ng/ml) compared to the AChR- patients ($N = 23$; median: 2037 ng/ml; $p = 0.1139$; Fig. 1D).

Table 1
Patient demographics.

MG patients characteristics	Serum (n = 73)	Plasma (n = 23)
Sex		
F	*41 (56.2 %)*	14 (60.9 %)
M	*32 (43.8 %)*	9 (39.1 %)
Mean age (y ± SD)	*59.8 ± 18.2*	*59.2 ± 17.7*
Serology		
AChR+	*50 (68.5 %)*	*21 (91.3 %)*
Seronegative	*23 (31.5 %)*	*2 (8.7 %)*
MG subtype		
EOMG	*37 (50.7 %)*	*14 (60.9 %)*
LOMG	*36 (49.3 %)*	*9 (39.1 %)*
MG-ADL (points)	*Median: 4 (Range 0–18, 95 % CI: 2–5)*	*Median: 4 (Range 0–13, 95 % CI: 3–8)*
MGC (points)	*Median: 4 (Range 0–34, 95 % CI: 3–7)*	*Median: 5 (Range 0–17, 95 % CI: 3–11)*
Immunosuppressive treatment		
No	*33 (38.5 %)*	*11 (47.8 %)*
Yes	*40 (61.5 %)*	*12 (52.2 %)*
Corticosteroids (n)	*30*	*7*
Azathioprine (n)	*12*	*4*
Cyclosporine (n)	*0*	*2*
Rituximab (n)	*3*	*3*
IvIg (n)	*4*	*1*
Tacrolimus (n)	*1*	*0*
Mycophenolate mofetil (n)	*1*	*0*

Abbreviations: F, female; M, male; AChR+, acetylcholine receptor antibody seropositive; EOMG, early-onset MG; LOMG, late-onset MG; MG-ADL, MG Activities of Daily Living; MGC, Myasthenia Gravis Composite score. Immunosuppression treatment may not add up to the number of patients receiving this due to patients having a combination of different treatments.

For the last subgroup comparison based on age at onset, there was no difference between 33 EOMG patients (median: 2593 ng/ml) and 40 LOMG patients (median: 2108 ng/ml, $p = 0.2307$; Fig. 1E) or between the 41 women (median: 2381 ng/ml) and 32 men (median: 2228 ng/ml, $p = 0.8460$; Fig. 1F).

In a cohort of 27 patients with longitudinal weekly samples and unchanged treatment, levels of C1s-INH were found not to differ significantly between the weeks ($p = 0.0793$).

3.2. Plasma levels of C1s-C1-INH levels are comparable in MG and in matched healthy controls

Plasma levels of C1s-C1-INH were assessed from 23 MG patients (9 men, mean age: 59.2 ± 17.7 years; Table 1) and 57 HCs (18 men, 51.7 ±

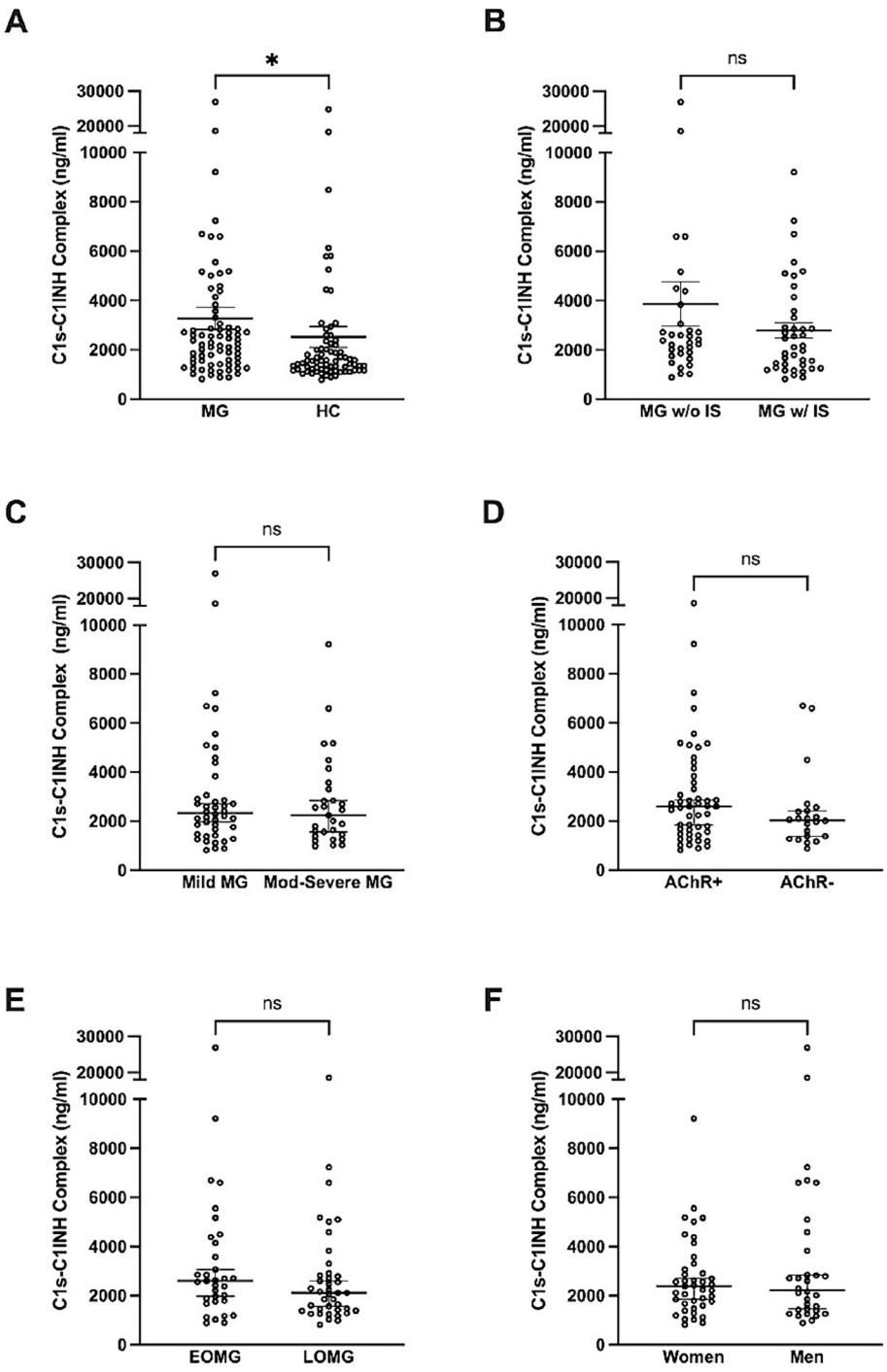

Fig. 1. Serum levels of C1s-C1-INH complex. A) Comparison between all MG patients ($N = 73$) and matched healthy controls (HC, $N = 74$). Comparison between B) MG patients without immunosuppression (w/o IS) and with immunosuppression (w/IS), C) mild and moderate to severe MG; D) acetylcholine receptor antibody seropositive (AChR+) and negative (AChR-) patients; E) early-onset MG (EOMG) and late-onset MG (LOMG) as well as F) women and men. * $p < 0.05$; n.s = non significant.

15.1 years, Table 1). The MGC scores varied from 0 to 17 points (median: 5 points; 95 % CI: 3–11 points), and MG-ADL scores ranged from 0 to 13 points (median: 4 points; 95 % CI: 3–8 points). C1s-C1-INH levels were elevated in MG patients' plasma (median: 1462 ng/ml; 95 % CI: 1290–1962 ng/ml) versus HCs (median: 1266 ng/ml; 95 % CI: 1189–1443 ng/ml; $p = 0.0214$; Fig. 2). C1s-C1-INH did not correlate with MGC ($r = -0.2310$; $p = 0.2888$) or MG-ADL($r = -0.3247$; $p = 0.1306$), and no correlation was observed between the plasma levels and serum levels of C1s-C1-INH ($r = 0.3449$; $p = 0.1071$).

4. Discussion

In this study, we analyzed the levels of C1s-CI-INH in MG patients and healthy controls and different subgroups of MG in both serum and plasma samples. We showed that C1s-CI-INH concentrations were higher in MG patients than in HCs, most likely due to activation of the early classical pathway. Quantifications of individual complement components, activation products, and complement activity in MG patients' blood samples have been reported in several studies to evaluate

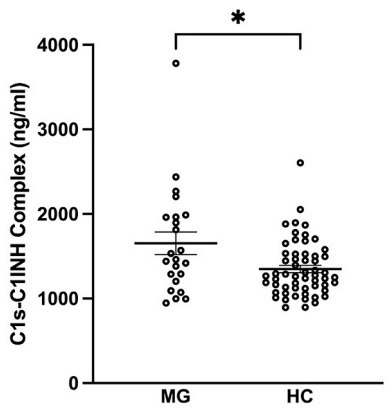

Fig. 2. Plasma levels of C1s-C1-INH complex. Comparison between all MG patients ($N = 23$) and matched healthy controls (HC, $N = 57$). * $p < 0.05$; n.s = non significant.

the complement status (Aguirre et al., 2020; Fichtner et al., 2022; Iacomino et al., 2022; Iwasa et al., 2023; Ozawa et al., 2021). Nevertheless, there is scarce information regarding the proximal components of the classical complement pathway in MG. The large proteolytic enzyme C1 complex has recently drawn attention since it is pivotal as the initial component that triggers proteolytic cascade upon complement activation (Mortensen et al., 2017). Evaluation of upstream components of the complement pathway, such as the C1 complex, in blood samples allows for detecting complement system involvement at an earlier stage and offers more specific information about the underlying immune processes. So far, the study of the upstream classical pathway in MG has solely involved the analysis of C1q, a subunit of the C1 complex (Iacomino et al., 2022).

Complement component 5 (C5) targeting therapies are clinically beneficial in patients with AChR+ generalized MG, clearly supporting their IgG1 antibody-mediated complement activation (Howard Jr. et al., 2023; Muppidi et al., 2019). Intriguingly, in this study, we also found that patients who were AChR antibody seronegative (AChR-) had comparable levels as AChR+ MG patients of C1s-CI-INH, supporting a possible activation of the classical pathway also in the AChR- subgroup. This finding was not unexpected since the complement depositions at the NMJ were also observed in AChR- MG patients, showing positive stainings of C1q, IgG1, and C5b-9 in skeletal muscle specimens (Hoffmann et al., 2020). Increased plasma levels of cleaved complement components, including C3a and C5a, are known to still be present in AChR-Ab+ patients under standard immunosuppressive therapies and not only in immunosuppressive naiive patients (Stascheit et al., 2023). These findings align with our data since serum and plasma data show early signs of classical pathway activation in patients with standard immunosuppressive treatment, including prednisone and azathioprine in most patients. The explanation for no significant difference in C1s-C1-INH levels between patients with and without immunosuppression might be due to the different effects of corticosteroids and azathioprine on complement factors. Corticosteroids reduce complement levels in serum, whereas azathioprine does not (Atkinson and Frank, 1973; Cavallo et al., 1984). Another study reported increased levels of C3 and C5a in AChR+ MG patient plasma, but no correlation was seen with disease severity (Iacomino et al., 2022). This also aligns with our study, where we could not detect any clear correlation between serum or plasma C1s-CI-INH and disease severity. Further, the complement activity in AChR+ MG patients was not associated with disease severity (Fichtner et al., 2022). Measurement of circulating complement levels, either serum or plasma, might not accurately reflect the actual complement-mediated damaged muscle tissues since complement activation occurs locally rather than systemically. The correlation between complement status and disease severity has also been investigated in systemic lupus erythematosus and several reasons have been inferred to explain why it is not easy to correlate disease severity with complement levels, e.g. wide variation in normal complement protein levels between different individuals (Walport, 2002).

In summary, the higher levels of C1s-CI-INH in the serum and plasma of MG patients indicate early activation of the classical pathway. These findings allow for prospective studies evaluating activated C1s as a putative treatment target and a potential accompanying biomarker in MG.

CRediT authorship contribution statement

Yu-Fang Huang: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Caitlin M. Briggs:** Writing – review & editing, Resources, Funding acquisition. **Sankalp Gokhale:** Writing – review & editing, Resources, Funding acquisition. **Anna Rostedt Punga:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Dianthus Therapeutics sponsored the study.

Data availability

All data supporting the findings of this study are available within the paper. The raw datasets with anonymized data will be shared upon reasonable request from any qualified investigator.

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