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Decreased serum IL-16 and increased serum TSG-14 levels in myasthenia gravis

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Abstract

Background: Myasthenia gravis (MG) is an autoimmune disease characterized by an imbalance between inflammatory mediators and immune cells. The diagnosis and assessment of disease severity require the aid of more specific biomarkers. *Methods:* Utilizing the Luminex assay, we quantified the serum levels of CD137, galectin-9, MMP12, TSG14, IL-16, IL-31, and IL-34 in a cohort of 157 participants, which comprised 98 patients with MG and 59 healthy controls (HCs). Correlation analyses were performed to examine the relationship between serum IL-16 and TSG-14 levels and clinical outcomes. *Results:* Serum levels of IL-16 were significantly lower in MG patients compared to the HC group (median [inter-quartile range (IQR)], 102.1 [73.58–126.7] pg/mL versus 162.4 [95.72–312.7] pg/mL, $P < 0.0001$). Conversely, the serum concentration of TSG-14 was significantly higher in MG patients than in HCs (1249 [809.3–2134] pg/mL versus 883.0 [512.5–1264] pg/mL, $P = 0.0035$). No significant differences were observed in the serum levels of CD137, galectin-9, IL-31, IL-34, and MMP12 between MG patients and HCs. Correlation analysis revealed a negative correlation between serum IL-16 levels and Quantitative Myasthenia Gravis (QMG) scores, as well as Activities of Daily Living (ADL) scores; TSG-14 displayed a positive correlation with QMG scores.
Conclusion: Serum IL-16 concentrations were reduced, whereas TSG-14 concentrations were elevated in MG patients. These findings suggest that these serum proteins can potentially serve as biomarkers for assessing disease severity in MG patients.

Keywords: Myasthenia gravis, IL-16, TSG-14, Luminex

INTRODUCTION

Myasthenia gravis (MG) is characterized by muscle weakness due to autoantibodies targeting the acetylcholine receptor (AChR), muscle-specific tyrosine kinase (MuSK), lipoprotein-related protein 4 (LRP4), or agrin, with approximately 85% of cases being seropositive for anti-AChR antibodies.[1] Besides typical clinical manifestations and serological tests, the diagnosis of MG is further supported by repetitive nerve stimulation, single-fiber electromyography (EMG), and the ice pack test.[2] The heterogeneity among patients necessitates additional specific biomarkers for accurate clinical diagnosis and severity assessment, paving the way for precision medicine. This study investigates seven immunologically relevant proteins (CD137, galectin-9, MMP12, TSG14, IL-16, IL-31, and IL-34), previously unreported in MG, to uncover valuable biomarkers for the condition.

CD137, expressed on activated T cells, serves as an effective co-stimulatory receptor. Soluble CD137 (sCD137) in the serum of patients with various autoimmune diseases acts as a natural regulator of immune responses, holding therapeutic potential.[3] Studies have demonstrated that serum CD137 levels are significantly elevated in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), correlating with disease severity, suggesting its role as a biomarker for disease activity.[4,5] Galectin-9 (Gal-9), a β-galactoside-binding lectin, is expressed across all organ systems and is known for its immunoregulatory role in various microbial infections. Gal-9 is involved in numerous physiological processes including cell growth,

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differentiation, adhesion, communication, and death.[6] Recent studies have highlighted increased levels of Gal-9 in several autoimmune diseases, such as in the serum and cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients, as well as in SLE and systemic scleroderma (SSc) patients.[7–10] MMP12, a matrix metalloproteinase (MMP), also known as a metalloproteinase, degrades proteins in the presence of active metal ions and is primarily secreted by macrophages. MMPs, including more than 20 types, break down elastin and various extracellular matrix (ECM) components, facilitating cell migration, and are upregulated in cancer and inflammation. Previous literature indicates increased plasma/serum concentrations of MMP-2,3,9,10 in patients with MG compared to healthy individuals.[11] Although MMP-12 has not been reported in MG, studies have shown patients with SSc and RA exhibit increased plasma MMP12 levels compared to controls.[12,13] TSG-14 (TNF-inducible gene 14), also known as PTX3, is a tumor necrosis factor and IL-1 inducible protein and a member of the acute-phase protein pentaxin family.[14] TSG-14, secreted by various cell types, especially during inflammatory responses, has been found in increased levels in the serum/plasma of patients with initial SSc, RA, and SLE compared to healthy controls.[13,15–18]

IL-16, initially recognized as a lymphocyte chemoattractant, is a pro-inflammatory cytokine produced in its precursor form (pro-IL-16) and activated by caspase-3 processing. Its primary sources include $CD4^+$ T cells, $CD8^+$ T cells, eosinophils, and dendritic cells.[19] Elevated levels of IL-16 have been detected in various autoimmune diseases, including the plasma, urine, and kidney tissue of SLE patients[20–22], and cerebrospinal fluid of MS patients.[23] IL-31, a pro-inflammatory cytokine belonging to the IL-6 cytokine family, is typically associated with the activation and differentiation of T cells, particularly in Th2-type immune responses. Previous studies have shown that the IL-33/IL-31 axis is a potential inflammatory pathway in allergic and inflammatory diseases, with both IL-31 and IL-33 expression correlated to disease severity.[24] Previous research has reported significantly increased expression of IL-33 in the serum of patients with MG, correlating positively with the Quantitative Myasthenia Gravis (QMG) score.[25] The role of IL-31 in MG remains unreported. IL-34 shares a common receptor with macrophage colony-stimulating factor (M-CSF) and binds to CSF-1R, inducing lymphocyte differentiation, proliferation, and regulation of inflammatory component synthesis.[26] Several studies have reported aberrant expression of IL-34 in various autoimmune diseases, such as SLE[27], RA[28], and SSc.[29]

These proteins play vital roles in the immune system and have been proven to serve as biomarkers for disease activity in various conditions. However, their levels and potential mechanisms in MG have never been reported, indicating their significant potential as biomarkers for diagnosing and prognosticating MG. The advancement of Luminex technology allows for the simultaneous quantitative analysis of multiple proteins with minimal sample use, making it an ideal method for biomarker screening. This study employs Luminex multiplex analysis technology for application in MG and other autoimmune diseases.

METHODS

Participants

This study enrolled patients diagnosed with MG and age- and sex-matched healthy controls (MG group, $n = 98$; healthy control (HC) group, $n = 59$) from the Department of Neurology at the First Affiliated Hospital of Chongqing Medical University. Inclusion criteria for MG participants were as follows: (a) aged 18 years or older; (b) seropositive for acetylcholine receptor (AChR) antibodies; (c) no immunosuppressants or steroids used within the last three months; and (d) absence of other autoimmune, inflammatory, or infectious diseases. Baseline clinical characteristics of the participants are detailed in Table 1. Fasting blood specimens were collected, left to clot at ambient temperature for two hours, and centrifuged to separate serum.

Serum proteins measurement using Luminex

Serum levels of CD137, galectin-9, MMP12, TSG14, IL-16, IL-31, and IL-34 were quantified employing a Human Premixed Multi-Analyte Kit (R&D Systems, cat. LXSAHM-09), adhering to the manufacturer's protocols (H-Wayen Biotechnologies).

Statistical analysis

Statistical analysis was executed using GraphPad Prism version 9.0. Continuous variables were described as median [interquartile range (IQR)], with group differences assessed using the Mann-Whitney *U* test. Categorical variables were

Table 1: Baseline clinical characteristics of subjects

Variable	MG, n = 98	HC, n = 59	P-value[1]
Age, median (IQR), years	44.00 (27.25 – 57.00)	33.00 (28.50 – 37.50)	0.45
Gender, n (%)			
Female	51 (52)	30 (51)	0.89
Male	47 (48)	29 (49)	
MGFA at first sampling, n (%)			
I	24 (24)		
IIa	25 (26)		
IIb	22 (22)		
IIIa	5 (5.1)		
IIIb	19 (19)		
IVa	0 (0)		
IVb	3 (3.1)		
Thymoma, n (%)	14 (14)		
AChR-Ab positive, n (%)	98 (100)		

[1] Mann Whitney U test; Pearson's Chi-squared test
HC, healthy control; MG, Myasthenia gravis, MGFA, Myasthenia Gravis Foundation of America

reported as count (percentage) and analyzed using the Chi-square test or Chi-square test with Yates' correction as appropriate. Correlation between serum protein levels and clinical parameters in the MG cohort was determined using Spearman's method. Multiple linear regression analyses were further performed to explore associations. All tests were two-sided, and statistical significance was set at $P < 0.05$. Data points for proteins below detection limits were excluded from statistical evaluations.

RESULTS

Serum Levels of IL-16, CD137, galectin-9, TSG-14, IL-31, IL-34, and MMP-12 in patients with MG and Healthy Controls.

Protein concentrations too low for detection via Luminex were noted. As depicted in Figure 1A, in our study, serum detectability rates for IL-16, CD137, and galectin-9 were 100% across both MG and HC groups. The detectability rates for the other four proteins varied between groups but showed no significant differences: TSG-14 was detectable in 94.90% of MG versus 96.61% of HC participants ($P = 0.917$); IL-31 in 75.51% of MG versus 83.05% of HC ($P = 0.2666$); IL-34 in 76.53% of MG versus 81.36% of HC ($P = 0.4771$); MMP-12 in 88.78% of MG versus 94.92% of HC ($P = 0.3085$). Therefore, undetectable values were excluded from subsequent statistical analyses.

It was found that the serum concentration of IL-16 was significantly lower in patients with MG compared to the HC group (median [IQR], 102.1 [73.58–126.7] pg/mL vs. 162.4 [95.72–312.7] pg/mL, $P < 0.0001$); serum TSG-14 levels were significantly higher in patients with MG than in HCs (1249 [809.3–2134] pg/mL vs. 883.0 [512.5–1264] pg/mL, $P = 0.0035$) (Figure 1B). Due to the presence of several MG patients with exceptionally high serum TSG-14 levels (n = 7, >5000 pg/mL), we performed a comparative analysis against other patients (n = 86, TSG-14 < 5000 pg/mL). This revealed that patients with higher TSG-14 levels had significantly higher ADL and QMG scores (Supplementary Figure 1). No significant differences were observed in serum levels of CD137, galectin-9, IL-31, IL-34, and MMP-12 between the MG and HC groups as shown in Figure 1B.

Analyses of IL-16 and TSG-14 serum levels in various subgroups of patients with MG

Subgroup analyses performed in our study revealed no significant differences in serum levels of IL-16 and TSG-14 between thymoma-associated MG (TAMG, $n = 14$) and non-thymoma-associated MG (Non-TAMG, $n = 84$) groups. To explore this specific subgroup of MG patients with thymoma, we further analyzed the correlations between serum IL-16 and TSG-14 levels with clinical scores (ADL and QMG) in these 14 patients. No significant differences were found, as shown in Supplementary Figure 2. The patients were further categorized based on onset ages into two groups: early-onset MG (EOMG, onset < 50 years, $n = 63$) and late-onset MG (LOMG, onset ≥ 50 years, $n = 35$). The analysis indicated no significant difference in serum IL-16

A

Detection rates of 7 proteins in human serum

	IL-16	CD137	Galectin-9	TSG-14	IL-31	IL-34	MMP-12
MG	100%	100%	100%	94.90%	75.51%	76.53%	88.78%
HC	100%	100%	100%	96.61%	83.05%	81.36%	94.92%
P-value	-	-	-	0.917	0.2666	0.4771	0.3085

B

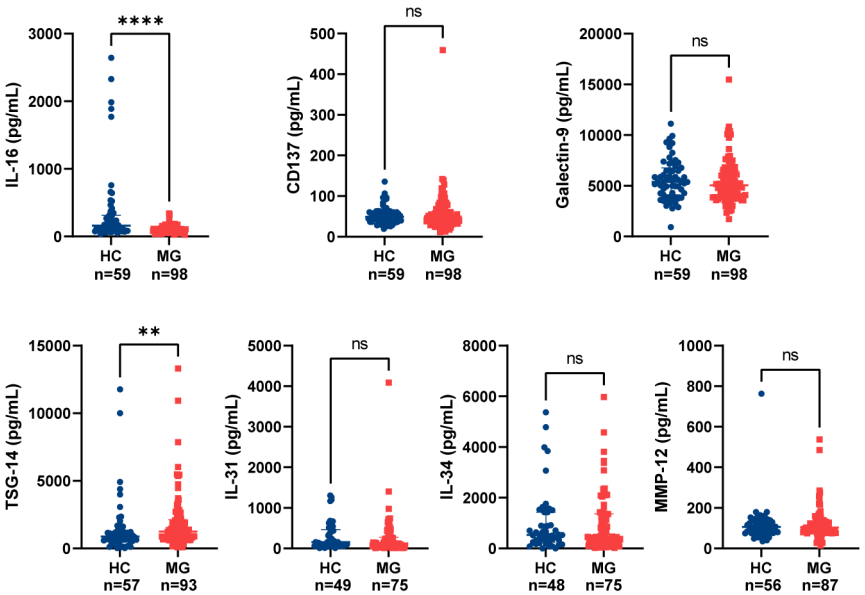


Figure 1. Serum Levels of IL-16, CD137, Galectin-9, TSG-14, IL-31, IL-34, and MMP-12 in patients with MG and healthy controls. (A) Comparison of detection rates of 7 proteins in human serum. (B) 7 proteins levels in patients with MG compared to the HCs. HC, healthy control; MG, Myasthenia gravis. **P < 0.01 and ****P < 0.0001.

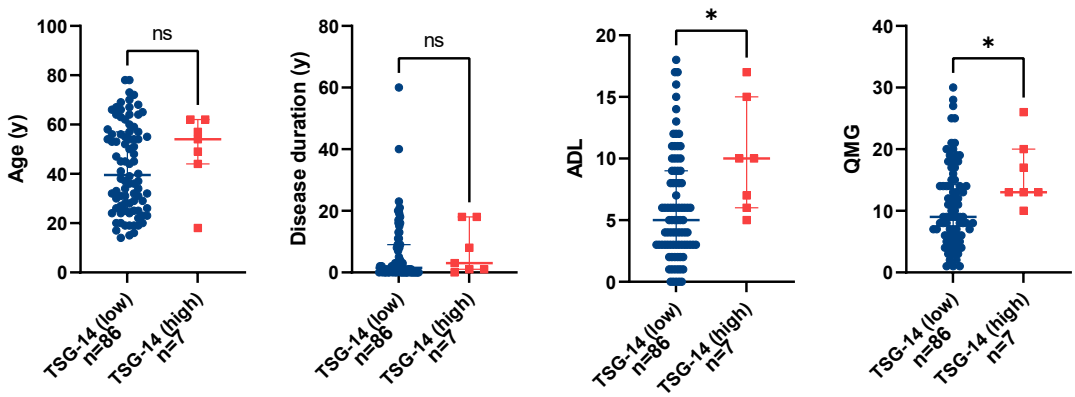
and TSG-14 levels between EOMG and LOMG patients. Additionally, patients with MG were classified into short disease duration (≤ 6 months) and long disease duration (> 6 months) groups, revealing no disparity in serum IL-16 and TSG-14 levels between these duration groups in our study.

Furthermore, based on the distribution of muscle involvement, patients were divided into ocular MG (OMG) and generalized MG (GMG) types. It was observed that serum IL-16 levels were significantly lower in the GMG group compared to the OMG group (median [IQR], 93.99 [64.52–118.3] pg/mL vs. 121.5 [90.14–144.7] pg/mL, *P* = 0.003). Although TSG-14 levels showed an increasing trend in the GMG group, the difference was not statistically significant (median [IQR], 1377 [877.9–2691] pg/mL vs. 1166 [846.6–1847] pg/mL, *P* = 0.305). When subdividing GMG patients according to the Myasthenia Gravis Foundation of America (MGFA) classification into MGFA II and MGFA III/IV groups, our study found no significant differences in serum IL-16 and TSG-14 levels between these two categories (Figure 2).

Correlation analyses of serum IL-16 and TSG-14 with clinical outcomes

We examined the associations of IL-16 and TSG-14 serum levels with various clinical parameters, including age, disease duration, quantitative MG test (MG-QMG), and MG activities of daily living (MG-ADL) score. The analysis revealed that serum IL-16 levels were inversely correlated with QMG scores (r = -0.2346, *P* = 0.0201) and MG-ADL scores (r = -0.2158, *P* = 0.0328), showing no correlation with age or disease duration (Figure 3A). Conversely, serum TSG-14 levels exhibited a direct correlation with QMG scores (r = 0.2078, *P* = 0.0457) but were not associated with MG-ADL scores, age, or disease duration (Figure 3B).

Multiple linear regression analyses demonstrated that IL-16 levels independently negatively affected QMG scores (*P* = 0.0091) and MG-ADL scores (*P* = 0.0109). In contrast, TSG-14 levels independently positively impacted QMG scores (*P* = 0.0346) and MG-ADL scores (*P* = 0.0310) (Table 2).



	Male	Female	P-value
TSG-14 (high)	3	4	
TSG-14 (low)	41	45	0.8822

	TAMG	Non-TAMG	P-value
TSG-14 (high)	2	5	
TSG-14 (low)	12	74	0.6238

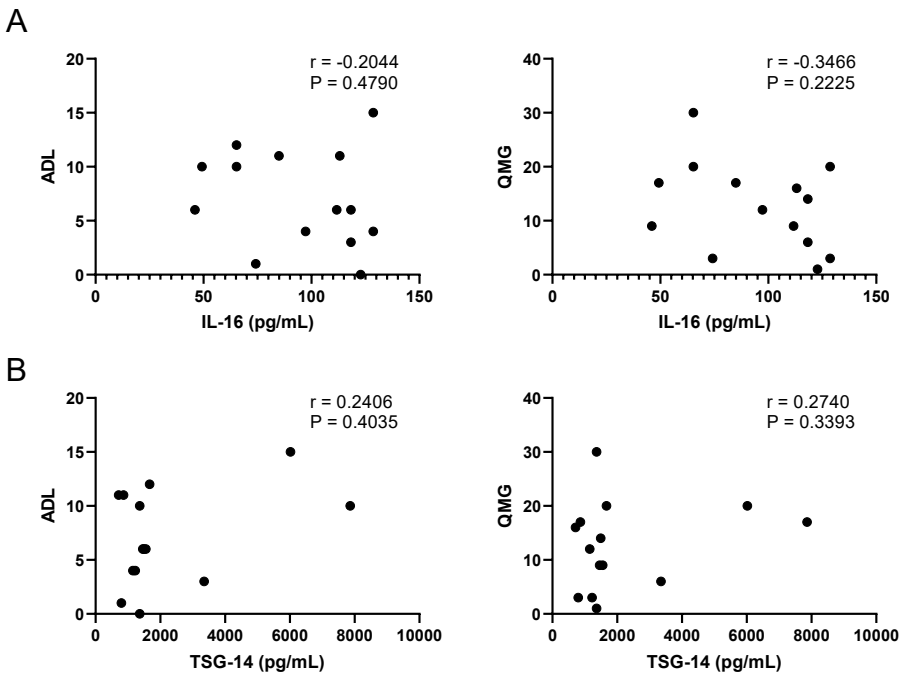
Supplementary Figure 1. Comparison of Clinical Characteristics (Age, Sex, Disease Duration, Thymoma, ADL, and QMG Scores) Between Myasthenia Gravis Patients with Serum TSG-14 Levels Above and Below 5000 pg/mL.

DISCUSSION

MG is an autoimmune condition where the dysregulation of immune cells and proteins plays a critical role. Clinically, a comprehensive assessment of various immune cell ratios and protein levels is essential for accurate diagnosis and prognosis of MG. Although several biomarkers have been identified in MG, unexplored immunoproteins with significant potential merit further investigation. In this context, we utilized Luminex technology to measure seven immune-related proteins in the serum of patients with MG, identifying TSG-14 and IL-16 as potential biomarkers for the disease.

TSG-14, secreted by various cell types during inflammatory responses, has been found elevated in multiple autoimmune diseases, consistent with our findings of increased serum TSG-14 levels in patients with MG. Additionally, TSG-14 levels positively correlate with QMG scores, indicating its pro-inflammatory role in MG. However, within the complex human immune environment, TSG-14 does not consistently promote inflammation. For instance, studies have demonstrated that PTX3 stimulation of human peripheral blood mononuclear cells (PBMCs) in vitro increases the production of the anti-inflammatory cytokine IL-10, while not affecting the production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α.[30] Moreover, PTX3 can bind to MD-2, activating the anti-inflammatory TLR4/TRIF signaling pathway, thereby mitigating the inflammatory burden following fungal infections.[31]

Similarly, elevated levels of IL-16 have been found in a variety of other autoimmune diseases. Past research underscores IL-16's pro-inflammatory role, contributing to the exacerbation of diseases like SLE and RA, with reductions in IL-16 linked to mitigated disease severity.[32] However, in this study we found that serum IL-16 levels were significantly decreased in the MG group and showed a negative correlation with clinical scores, suggesting that it may play a potential protective role in MG. This is not the first study to suggest a potential protective role for IL-16 in autoimmune diseases, such as a



Supplementary Figure 2. Correlation Analysis Between Serum IL-16 and TSG-14 Levels and Clinical Scores (ADL and QMG) in Myasthenia Gravis Patients with Thymoma.

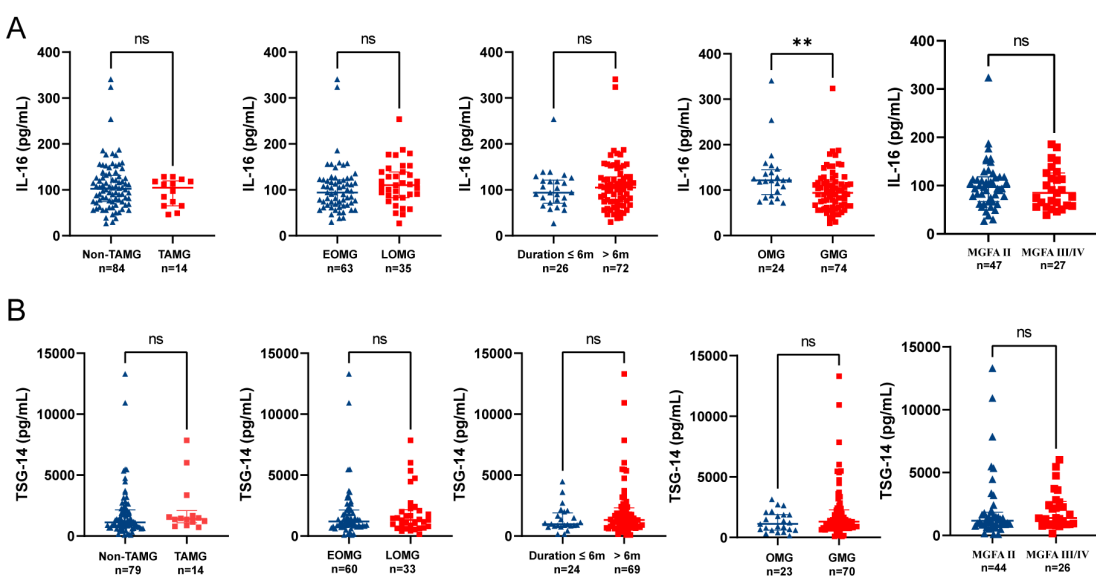


Figure 2. Analyses of IL-16 and TSG-14 serum levels in various subgroups of patients with MG
Serum IL-16 (A), TSG-14 (B) levels in subgroups stratified by thymoma, onset age (onset age < 50 years versus onset age ≥ 50 years), disease duration (duration ≤ 6 months versus duration > 6 months),muscle group involvement (OMG versus GMG), and MGFA classifications (GMG with MGFA II versus GMG with MGFA III/IV). EOMG, early-onset MG; GMG, generalized MG; LOMG, late-onset MG; MG Myasthenia gravis; MGFA, Myasthenia Gravis Foundation of America; OMG, ocular myasthenia gravis; TAMG, thymoma-associated MG. **P < 0.01.

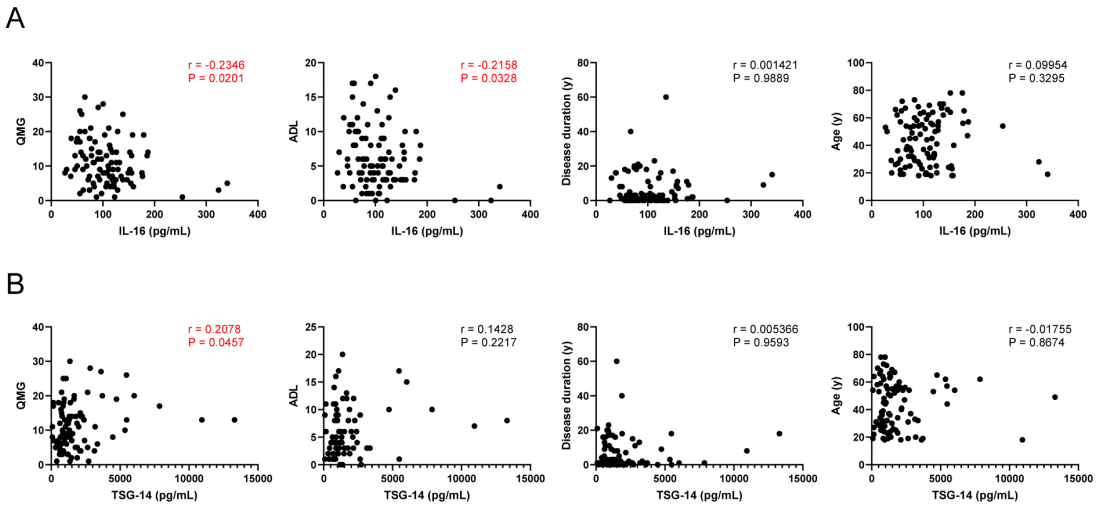


Figure 3. Correlation analyses of serum IL-16 and TSG-14 with clinical outcomes
Correlation analyses of serum IL-16 (A) and TSG-14 (B) with QMG, ADL, disease course and age. ADL, activities of daily living; QMG, quantitative MG test.

study suggesting that IL-16 may confer a benefit in RA disease, as treatment of synovium-severe combined immunodeficiency (SCID) mice with recombinant IL-16 decreased the production of inflammatory cytokines.[33] These findings reveal a bidirectional role of IL-16 in various diseases.

Initially described as a chemoattractant for $CD4^+$ T cells, IL-16 regulates T cell activation, IL-2 expression, and immune synapse formation, indicating its critical role in diseases associated with $CD4^+$ T cells.[19] Furthermore, its involvement in recruiting eosinophils and dendritic cells highlights its broader immunological role.[19] Despite its association with pro-inflammatory activity in various autoimmune and inflammatory diseases, IL-16's negative correlation with disease severity in MG suggests a protective mechanism worth further exploration, especially considering Treg cells are known to be defective in number and function in MG.[34–36] Notably, previous studies indicate that IL-16 can directly induce the expression of FoxP3 and enhance the functionality of regulatory T cells, thereby expanding the inducible Treg population.[37,38] These findings suggest that IL-16 may exert a unique protective role in MG by modulating the quantity and functionality of Treg cells.

While significant changes in serum levels of IL-16 and TSG-14 have been identified in patients with MG, the other five proteins studied did not exhibit any significant differences between the MG and HC groups. Despite these proteins being active in various autoimmune diseases, our findings suggest a unique immunological environment in MG, distinct from other autoimmune conditions. This discovery prompts further exploration for MG-specific biomarkers, aiming for precise therapeutic and preventative strategies. Notably, generalized MG patients often present with more severe conditions than those with ocular MG. Our subgroup analyses showing lower serum IL-16 levels in GMG patients, indicating a potential correlation between decreased IL-16 levels and increased disease severity.

Table 2: Multiple linear regression analyses of the effect from serum proteins on the clinical outcomes

Variables	QMG	ADL
IL-16 (pg/mL)	-0.03401 (0.0091)	-0.02215 (0.0109)
TSG-14 (pg/mL)	0.0006835 (0.0346)	0.0004668 (0.0310)
n	93	93

Dependent variable: QMG or ADL
Independent variables: IL-16 and TSG-14
Unstandardized regression coefficient (P-value) was given
ADL, activities of daily living; QMG, quantitative MG test.

Clinical assessments of MG severity can vary due to multiple factors affecting evaluators and patients, leading to inconsistent results. In this study, serum IL-16 levels showed a significant negative correlation with clinical severity scores (QMG and ADL), while TSG-14 correlated positively with QMG scores. Multiple linear regression analyses confirmed these proteins' significant impact on these scores, indicating that serum IL-16 and TSG-14 levels could objectively determine disease severity in clinical practice.

Several limitations in this study need to be addressed. This study included only acetylcholine receptor antibody-positive MG patients who had not used immunosuppressive drugs, which leaves uncertainty about how other types of autoantibodies or the use of immunosuppressants might influence serum levels of IL-16 and TSG-14. Future studies with larger and more diverse MG patient cohorts are necessary to comprehensively evaluate the role of IL-16 and TSG-14 in MG. Additionally, this cross-sectional analysis of serum protein levels in MG necessitates longitudinal cohort studies to validate the predictive capability of serum IL-16 and TSG-14 levels for MG prognosis.

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DISCLOSURE

Ethics: This investigation was sanctioned by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (2020-382, NCT04674605), and all participants provided written informed consent.

Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest: None.

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