# Calprotectin as potential novel biomarker in myasthenia gravis

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## A B S T R A C T

Myasthenia gravis (MG) is the most common autoimmune disease affecting the neuromuscular junction by specific autoantibodies. The etiology of MG and its heterogeneity in clinical courses are poorly understood, although it was recently shown that gut microbial dysbiosis plays a critical role. Since levels of Calprotectin (CLP) seem to correlate with level of dysbiosis, we hypothesize that CLP may serve as potential disease activity biomarker in MG. Sera from 251 patients with MG and 90 controls were analyzed in an explorative, cross-sectional design. Prospectively, we tested CLP levels in MG patients up to 3 years. Association of CLP levels with socio-demographics, disease activity (quantitative myasthenia gravis (QMG) score, myasthenia gravis-specific Activities of Daily Living scale (MG-ADL)), antibody (Abs) status, history of myasthenic crisis, treatment regime, and history of thymectomy were investigated using univariate analysis. Mean baseline serum levels of CLP were significantly higher in MG patients compared to controls (4.3 μg/ml vs. 2.1 μg/ml; $p < 0.0001$). Higher levels of CLP were associated with a higher clinical disease severity measured by MGFA classification and QMG score. Nevertheless, the only weak correlation of CLP with clinical outcome parameters needs confirmation in future studies. Currently, there are no validated blood biomarkers for MG. The significantly elevated CLP and mild correlation with parameters of disease activity suggests that CLP holds promise as a biomarker for measurement of individual disease severity.

## 1. Introduction

Myasthenia gravis (MG) is an autoimmune disease affecting the neuromuscular junction by specific autoantibodies [1,2]. While the final pathways of the disease and its effectors disturbing the functions of the neuromuscular junction are relatively well known, the etiology of MG and its heterogeneity in clinical course are poorly understood. Importantly, there is an urgent need for a sensitive biomarker in MG that reliably predicts the individual disease course and exacerbation, as well as guiding immune suppressive treatment, especially in the light of emerging and more specific therapy options for MG patients [3].

Both, genetic and environmental factors have been considered crucially involved in the etiology of MG [4]. While the exact factors responsible for predisposition to MG remain elusive, a crucial role for gut microbiota in the pathogenesis of MG has been hypothesized, since MG patients show a high level of microbial dysbiosis [5–7].

Similarly to inflammatory bowels diseases (IBD) [8], the incidence of MG is increasing in newly industrialized countries [9,10], supporting further an association between "westernization of lifestyles", gut microbiome and MG.

Calprotectin (CLP), a calcium-binding protein of the S100 family, performs various biological functions via interaction with Toll-like receptor 4 [11] on the surface of leukocytes and is manly released by activated neutrophils, monocytes and early differentiation states of macrophages [12]. CLP has been shown to perform various biological functions, especially in triggering signaling pathways involved in inflammatory processes and inhibition of microbial growth [12]. In IBD, levels of CLP not only correlated with the level of microbial dysbiosis

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[13], but were also significantly increased in patients with active disease [14] and strongly predicted disease relapse and treatment response [15]. CLP was also investigated in other autoimmune diseases, mainly rheumatoid arthritis (RA) [16], where prediction of disease activity and treatment outcome was proven high. Of highlight, CLP leads to the release of pro-inflammatory cytokines [17,18] and induction of auto-reactive CD8+ T cells [18,19], which play a key role in the pathogenesis of MG [20].

Therefore, we want to test the hypothesis that CLP may serve as a potential disease activity biomarker using a cohort of MG patients compared to controls.

## 2. Methods

### 2.1. Standard protocol approvals, registrations, and patient consent

The study was approved by the ethics committee of the Charité-Universitätsmedizin Berlin (EA1/281/10). All patients gave written informed consent in accordance with the Declaration of Helsinki in its currently applicable form.

### 2.2. Study design

This is an explorative cross-sectional and prospective study comparing serum CLP levels of MG patients and controls to assess the potential of CLP to measure disease activity as assessed by MGFA classification system, quantitative myasthenia gravis (QMG) score, and myasthenia gravis-specific Activities of Daily Living scale (MG-ADL).

### 2.3. Patients and controls

This study was performed at the certified integrated Center for Myasthenia gravis (iMZ) of the Charité-Universitätsmedizin Berlin, Germany. Patients over the age of 18 years with confirmed diagnosis of myasthenia gravis based on the current guidelines of the German Neurological Society [21] were included independent of disease duration and severity. Overall, 251 patients were consecutively screened at the iMZ clinic between March 2016 and May 2020 and were further categorized in subgroups according to age at onset (early-onset MG [EOMG] was defined as onset at ≤50 years of age, late-onset MG was defined as onset >50 years of age [22]) and thymus pathology (thymoma-associated MG [TAMG]). Prospectively, we tested CLP levels in an explorative design in a limited cohort of 58 MG patients over 3 years.

Socio-demographics (age, sex, disease duration), history of myasthenic crisis, antibody status (acetylcholine receptor antibody [AChR-Abs], muscle specific receptor tyrosine kinase antibody [MuSK-Abs], lipoprotein-related protein 4 [LRP4], seronegative), current MG specific medication (cholinesterase inhibitors, glucocorticoids, and long-term immunosuppressant's), history of thymectomy, and comorbidities were collected in a database. Exclusion criteria were age <18, previous history of cancer except thymoma [23], and diagnosis of RA or IBD due to potential influence on CLP levels. 77 age and gender matched voluntary HC were enrolled as a healthy control group, as well as 13 patients with non-inflammatory neurological diseases (NC) recruited from the outpatient clinic for polyneuropathies as a diseased control group. Exclusion criteria for controls were history of autoimmune disorders, neurological diseases other than polyneuropathies, obesity, cardiovascular diseases as well as history of cancer.

### 2.4. Clinical assessment

Clinical outcome was assessed using the MGFA classification for disease classification [24] and the QMG score for disease severity. Using the MGFA classification, patients were grouped into remission (MGFA 0), ocular (MGFA I) or generalized MG patients (MGFA II-IV) at time of study inclusion and blood sampling. We have not used the MGFA

classification by employing the most severely affected muscles of disease history but for current disease severity to define the patient's MGFA class [24].

The QMG score was developed as a tool for assessing disease severity as well as the pattern of deficits based on quantitative testing of sentinel muscle groups [24,25]. Its reliability and validity have been demonstrated in several studies [25,26]. QMG scores were assessed at baseline and follow up to evaluate disease severity. Moreover, patient reported outcome regarding impact on daily living was assessed at both corresponding time points using the MG-ADL [27,28].

### 2.5. Laboratory testing

Blood samples were collected from patients with MG and controls immediately centrifuged and stored at −80 °C until being analyzed in May 2020. Serum levels of CLP were measured using the fCAL turbo method® on a COBAS 8000 semi-automated analyses (Bühlmann Laboratories AG, Schönenbuch, Switzerland) according to manufacturer's protocol [29,30]. The fCAL turbo method® has been validated for accurate measurement of CLP levels in serum [29].

### 2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 8.2.1, GraphPad Software, San Diego, CA, USA) and SPSS (version 25; SPSS Inc., Chicago, USA). Continuous data are presented as means and standard deviation (SD) and categorical variables as absolute frequencies and percentages. Baseline serum levels of CLP between MG patients and HC were compared using the one-way analysis of variance (ANOVA) with corrections for multiple comparisons. Correlation between CLP levels, clinical, and laboratory assessments were examined using nonparametric Spearman correlation analysis. A univariate analysis was performed using Mann-Whitney nonparametric test to analyze the differences between groups, and the Kruskal-Wallis test was used to analyze the differences between three or more groups. To illustrate the predictive performance of CLP in regard to disease severity as measured by QMG and MG-ADL, we calculated a delta for changes in QMG, MG-ADL and CLP levels, performed a correlation using Spearman correlation coefficient as well as a Mann-Whitney test. A *p*-value<0.05 was considered statistically significant.

### 2.7. Data availability

Anonymized data will be shared upon reasonable request from a qualified investigator.

## 3. Results

### 3.1. Demographics and baseline characteristics of MG patients

Overall, we included 251 patients with MG, 77 HC and 13 NC (Table 1) and for prospective analysis 58 MG patients (Table 2). Mean age was 54.4 years (SD 17.4), 147 (59 %) were female. Median disease duration was 4.0 years (2.0–10.0). Mean age at disease onset was 46.1 years (SD 19.0). 208 (83 %) of MG patients were positive for AChR-Abs, 10 (3 %) for MusK-Abs, 0 for LRP4-Abs (0 %), and 34 (15 %) remained seronegative (SN). Disease severity at time of sampling ranged from MGFA class 0–IIIB (median II), mean QMG was 8.1 (SD 6.5) and mean MG-ADL was 4.9 (SD 3.9). 50 MG patients (20 %) had a history of myasthenic crisis as defined by rapid worsening of muscle weakness and potential airway compromise from ventilatory or bulbar dysfunction [31].

At time of sampling, 129 (52 %) of MG patients had already undergone a thymectomy, 56 MG patients (16 %) used symptomatic monotherapy with cholinesterase inhibitors, while the majority of patients additionally used oral corticosteroids (n = 75; 42 %) and/or steroid

**Table 1**
Baseline characteristics and medical history of MG patients and controls.

| | Total MG | EOMG | LOMG | TAMG | Controls | HC | NC |
|---|---|---|---|---|---|---|---|
| Demographics | 251 | 97 (39%) | 154 (61 %) | 30 (12 %) | 90 | 77 (86 %) | 13 (14 %) |
| Sex | 147 (59 %) | 77 (69 %) | 69 (45 %) | 19 (68 %) | 52 (58 %) | 46 (60 %) | 6 (38 %) |
| Female | | | | | | | |
| Age at diagnosis (YEARS) | 54.4 ± 1.4 | 36.1 ± 8.4 | 66.2 ± 9.9 | 58.1 ± 12.5 | 51.9 ± 11.8 | 47.2 ± 7.7 | 65.6 ± 10.9 |
| Disease duration (years) | 4.0 (2-0-10.0) | 4.0 (2.0–7.0) | 4.0 (2.0–11.3) | 3.5 (1.3–8.3) | – | – | – |
| History of myasthenic crisis | 50 (20 %) | 21 (22 %) | 27 (18 %) | 11 (39%) | – | – | – |
| MGFA classification at time point of sampling | 38 (15 %) | 20 (21 %) | 18 (12 %) | 0 (0 %) | – | – | – |
| *0* | 42 (17 %) | 13 (13 %) | 24 (16 %) | 5 (18 %) | | | |
| *I* | 144 (57 %) | 43 (44 %) | 80 (52 %) | 21 (75 %) | | | |
| *II* | 27 (11 %) | 13 (13 %) | 13 (8 %) | 1 (4 %) | | | |
| *III* | 0 (0 %) | 0 (0 %) | 0 (0 %) | 0 (0 %) | | | |
| *IV* | 0 (0 %) | 0 (0 %) | 0 (0 %) | 0 (0 %) | | | |
| *V* | | | | | | | |
| QMG- Score | 8.1 ± 6.5 | 8.1 ± 6.3 | 7.9 ± 6.5 | 9.4 ± 6.7 | – | – | – |
| MG-ADL- Score | 4.9 ± 3.9 | 5.0 ± 3.9 | 4.5 ± 3.7 | 4.6 ± 4.2 | – | – | – |
| History of thymectomy | 129 (52 %) | 66 (68 %) | 62 (40 %) | 28 (100 %) | – | – | – |
| MG-specific treatment at baseline | 56 (16 %) | 30 (31 %) | 32 (21 %) | 2 (7 %) | – | – | – |
| Cholinesterase inhibitors monotherapy | 75 (43 %) | 28 (29 %) | 83 (54 %) | 10 (36 %) | | | |
| Corticosteroids | 86 (29 %) | 22 (23 %) | 48 (31 %) | 12 (43 %) | | | |
| Azathioprine | 28 (11 %) | 9 (9 %) | 17 (13 %) | 2 (7 %) | | | |
| MycophenolatE mofetil | 12 (5 %) | 6 (6 %) | 6 (4 %) | 0 (0 %) | | | |
| Methotrexate | 10 (4.0 %) | 6 (6 %) | 3 (3 %) | 4 (14 %) | | | |
| Rituximab | 7 (3 %) | 2 (2 %) | 5 (3 %) | 0 (0 %) | | | |
| Eculizumab | | | | | | | |

Data are mean (SD) and n (%) for the baseline variables and median (IQR) for disease duration. Disease duration is the time from diagnosis until baseline. *Abbreviations*: EOMG = early onset myasthenia gravis, HC = healthy controls, IQR = interquartile range; LOMG = late onset myasthenia gravis; MG = myasthenia gravis; MGFA = Myasthenia gravis foundation of America classification; MG-ADL = MG-activity of daily life score; NC = non-inflammatory neurological controls, SD−standard deviation, TAMG = thymoma - associated myasthenia gravis, QMG = quantitative myasthenia gravis score.

**Table 2**
Demographical and clinical characteristics of MG patients of prospective analysis.

| | V1* | V2** |
|---|---|---|
| Demographics | 58 | 58 |
| Sex | 23 (40 %) | 23 (40 %) |
| Female | | |
| Age at diagnosis (YEARS) | 57.6 ± 16.4 | 60.5 ± 16.1 |
| Disease duration (years) | 5.0 (1.8–14.0) | 8.0 (2.0–7.0) |
| History of myasthenic crisis | 8 (14 %) | 8 (14 %) |
| MGFA classification at time point of sampling | 6 (10 %) | 13 (22 %) |
| *0* | 16 (28 %) | 11 (19 %) |
| *I* | 33 (57 %) | 31 (54 %) |
| *II* | 2 (4 %) | 3 (5 %) |
| *III* | 0 (0 %) | 0 (0 %) |
| *IV* | 0 (0 %) | 0 (0 %) |
| *V* | | |
| QMG- Score | 6.6 ± 4.9 | 7.2 ± 5.7 |
| MG-ADL- Score | 4.2 ± 3.4 | 4.0 ± 3.7 |
| History of thymectomy | 26 (45 %) | 33 (57 %) |
| TYMOMYA | 6 (22 %) | 8 (24 %) |
| THYMITIS | 10 (39 %) | 10 (30 %) |
| WITHOUT PATHOLOGY | 10 (39 %) | 15 (46 %) |
| MG-specific treatment at Sample time | 7 (12 %) | 10 (17 %) |
| Cholinesterase inhibitors monotherapy | 8 (14 %) | 9 (16 %) |
| Corticosteroids | 12 (21 %) | 21 (36 %) |
| Azathioprine | 1 (2 %) | 8 (14 %) |
| MycophenolatE mofetil | 5 (9 %) | 9 (16 %) |
| Methotrexate | 1 (2 %) | 2 (3 %) |
| Rituximab | 0 (0 %) | 0 (2 %) |
| Eculizumab | | |

Data are mean (SD) and n (%) and median (IQR) for disease duration. Disease duration is the time from diagnosis until baseline. *Abbreviations*: IQR = interquartile range; MG = myasthenia gravis; MGFA = Myasthenia gravis foundation of America classification; MG-ADL = MG-activity of daily life score; SD−standard deviation, QMG = quantitative myasthenia gravis score, V1 = baseline visit, V2 = follow-up visit after 3 years.

sparing immunosuppressive therapy (n = 86; 29 %). In 17 MG patients (7 %), escalation therapies (rituximab; eculizumab) were required.

### 3.2. CLP levels are higher in MG

Baseline serum CLP levels were significantly higher in MG patients with a mean of 4.3 µg/ml (SD 3.0, 95 % CI 3.8–4.6) compared to HC (mean 2.1 µg/ml (SD 1.1, 95 % CI 1.2–2.2) and NC (mean 2.0 µg/ml (SD 1.2, 95 % CI 1.6–2.3); $p < 0.0001$; Fig. 1), with an area under the receiver operating curve (AUC) of 0.77 (95 % confidence interval (CI) 0.70–0.83; $p < 0.0001$). With a cut-off-value of 1.55 µg/ml CLP discriminated MG patients from controls with a sensitivity of 90.4 % and a specificity of 45.1 %.

There was a trend of highest levels in TAMG patients (4.9 µg/ml (SD 3.7, 95 % CI 3.4–6.2)), but not reaching statistical significance ($p = 0.218$, Kruskal-Wallis test).

Serum CLP levels in MG patients and controls were neither correlated to age ($r = -0.04$, $p = 0.459$) nor associated to gender ($p = 0.9246$; Mann-Whitney test).

Mean CLP levels in AChR-Abs positive patients (n = 208) were 4.2 µg/ml (SD 3.0), in MuSK-Abs positive patients (n = 10) 4.6 µg/ml (SD 4.1), and in seronegative MG patients (n = 34) 4.9 µg/ml (SD 2.9) showing no significant in between group differences ($p = 0.21$, Kruskal-Wallis test). Moreover, in AChR-Abs positive and MuSK-Abs positive MG patients, CLP levels did not correlate with Abs level ($r = 0.03$, $p = 0.677$ for AChR-Abs; $r = 0.04$, $p = 0.55$ for MuSK-Abs) (Spearman correlation coefficient). There was no significant difference in CLP levels in patients with (n = 51) and without (n = 200) a history of myasthenic crisis (n = 51) ($p = 0.213$, Mann-Whitney test).

### 3.3. Baseline CLP levels correlates with disease severity

Clinical severity measured by MGFA classification ranging from remission, I– III at time point of sampling were compared regarding CLP levels and revealed significant higher CLP levels in patients with a generalized compared with pure ocular MG or patients in remission ($p = 0.0435$, Kruskal-Wallis test, Fig. 2A). QMG score at baseline correlated
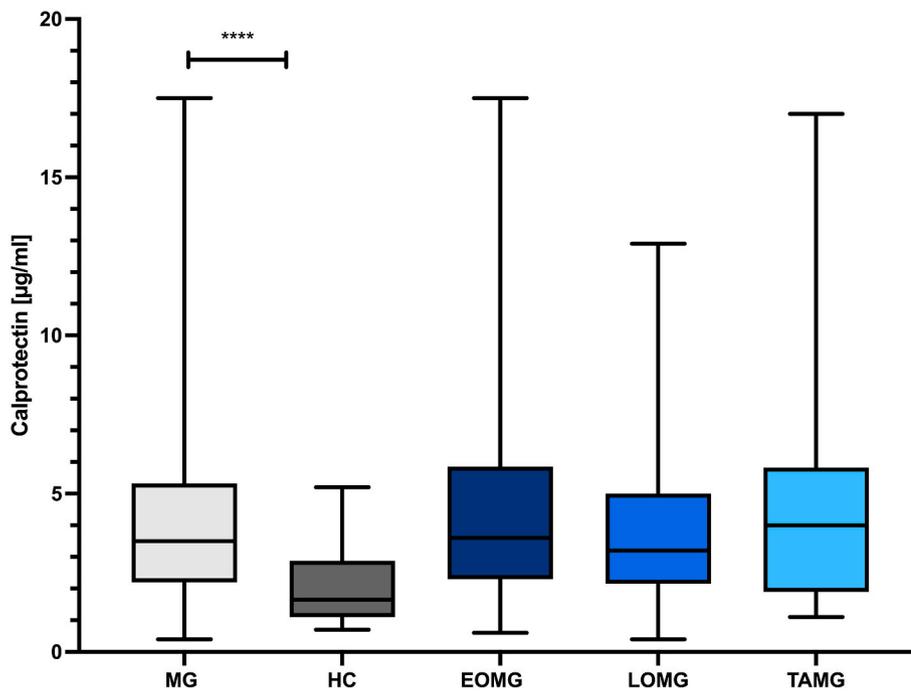
**Fig. 1.** Calprotectin levels are elevated in MG patients compared to controls. The box plot bar represents the mean baseline serum calprotectin (CLP) level with SD in all myasthenia gravis (MG) patients (n = 251) and subgroups compared to healthy controls (HC) (n = 77), n = number of patients with evaluable data. Mean CLP levels were significantly increased in all MG patients compared to HC regardless of MG-subtype (p < 0.001). Abbreviations: EOMG = early onset myasthenia gravis, LOMG = late onset myasthenia gravis; MG = myasthenia gravis; TAMG = thymoma - associated myasthenia gravis.

weakly, but significantly with serum CLP levels (r = 0.134, $p$ = 0.043, Spearman correlation coefficient, Fig. 2B). However, there was no significant correlation with the patient outcome parameter MG-ADL (r = 0.09, $p$ = 0.1664, Spearman correlation coefficient, Fig. 2C).

### 3.4. Relationship between CLP levels and treatment regime

Patients receiving only symptomatic monotherapy showed the highest levels of CLP at baseline (n = 75; 42 %; 4.1 μg/ml (SD 2.8)), whereas patients treated with eculizumab (n = 7 (3 %); 2.1 μg/ml (SD 0.4)) had the lowest, although not reaching statistical significance ($p$ = 0.072; Kruskal-Wallis test). MG patients with a history of thymectomy (at least >2 years) had similar mean baseline CLP levels compared to patients without history of thymectomy (4.3 μg/ml (SD 3.3) vs. 4.2 μg/ml (SD 2.7); Mann-Whitney test).

### 3.5. CLP not predictive with individual disease severity activity

To investigate the predictive performance of CLP regarding to individual changes in clinical (QMG) as well as patient reported outcome parameters (MG-ADL), we calculated a delta for each time point as well as a delta for individual change in CLP level and performed a Mann-Whitney test. We did not observe significant delta changes as defined by ≥ 2 points for QMG and ≥3 for MG-ADL in correlation with delta changes of CLP using Spearman correlation coefficient (r = 0.241 for QMG, r = 0.495 for MG-ADL). There was only a tendency for correlation in regard to changes over time for QMG score in total MG population at baseline (n = 251) and year 3 (n = 58) ($p$ = 0.410) (Fig. 3).

### 4. Discussion

This explorative cross-sectional and prospective study revealed that serum CLP levels of MG patients were significantly higher in comparison to controls. Moreover, baseline CLP levels correlated weakly, but positively with clinical disease activity as measured by QMG score and MGFA classification. Nevertheless, the only weak correlation of CLP with clinical outcome parameters needs confirmation in future studies.

It should be emphasized, that baseline CLP levels were elevated in all MG patients regardless of Abs status (AChR-Abs, anti-MuSK-Abs, SN),

which might be helpful in suspected MG cases, since about 15 % of the MG patients remain SN [1] and we have not found elevated CLP levels in the control group. Nevertheless, CLP is a sensitive but unspecific marker of inflammation. Its potential value therefore does not lie in the ability to discriminate different autoimmune diseases, but rather to reflect different degrees of disease activity, as proposed in other conditions like IBD [14] and RA [16,32], where CLP has become a routinely measured biomarker for disease severity. In addition, CLP has the potential to be a marker of microbial dysbiosis [13], which has been proposed to play a critical pathophysiological role in MG [7,33,34].

CLP leads to induction of auto-reactive CD8^{+} T cells, IL-17 [35] and other pro-inflammatory cytokines like IL-1β. The imbalance of inflammatory cytokines are involved in the pathogenesis of MG and play a central role in the development of inflammation at the neuromuscular junction [20,35]. IL-1β was reported to be a key cytokine which promotes Th17 cell generation, which is crucially involved in pathogenesis of MG [36]. In an experimental autoimmune MG mouse model, IL-17-knock out mice were developing fewer myasthenic symptoms and less pathogenic AChR-specific Abs [35]. Furthermore, increased IL-17 levels have been observed in MG patients [36], and an increased frequency of IL-17- producing CD4^{+} T cells has been demonstrated in particular for TAMG [37], which might explain the trend towards higher mean CLP level in TAMG patients in our cohort.

CLP was elevated in MG patients with high disease activity as scored with MGFA and QMG, which can effectively reflect the severity of the disease. This important finding relates to studies of CLP in IBD and RA, where CLP is routinely used as a disease activity and treatment response marker, especially for biologicals [15,38]. Although not statistically significant, this fact is further supported by the finding of lowest CLP levels in patients receiving eculizumab, providing additional evidence of the strong therapeutic efficiency of complement inhibition [39].

Socio-demographic parameters, history of myasthenic crisis, as well as Abs-status and levels had no effect on CLP levels, although several longitudinal studies in RA patients observed higher CLP levels in patients being positive for rheumatoid factor [16]. In addition, patients with a history of thymectomy >2 years showed no relevant difference regarding CLP level, which might be due to the lower classification regarding MGFA- and QMG-score at time of sampling in comparison to time of diagnosis.
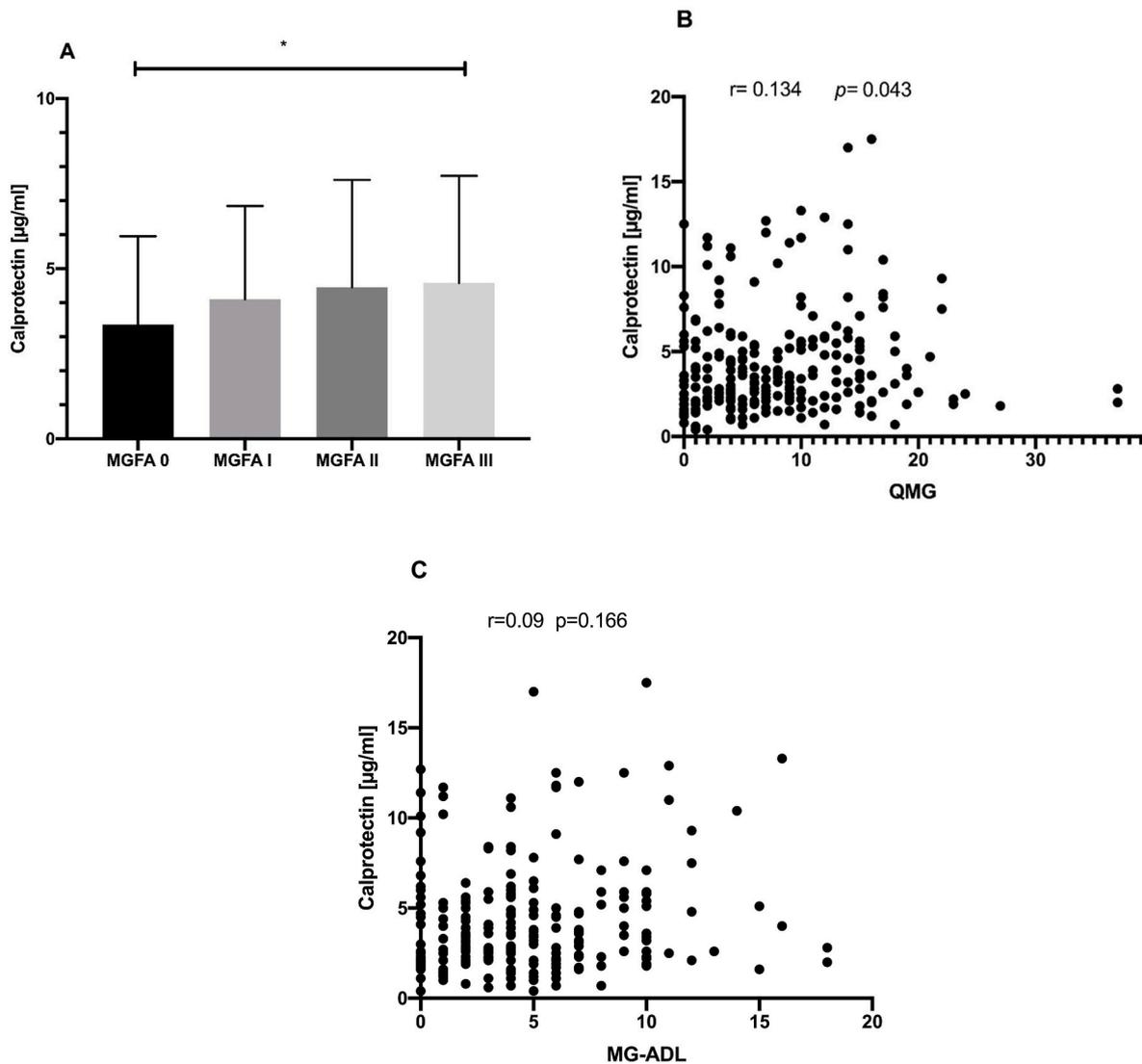
**Fig. 2.** Baseline CLP and clinical disease severity. **A:** Column bar graph of association of CLP level with clinical severity measured by Myasthenia gravis foundation of America classification (MGFA) using Kruskal-Wallis test. **B:** Correlation analysis of quantitative myasthenia gravis (QMG) score with serum level of CLP using Spearman correlation coefficient. **C:** Correlation analysis of myasthenia gravis activity of daily life (MG-ADL) score with serum level of CLP using Spearman correlation coefficient.

In the longitudinal analysis, the individual disease activity did not show a significant correlation in regard to delta changes. However, mean changes in primary outcome parameters QMG and MG-ADL were not significantly differing over the observed follow up time in our cohort, which might be mainly due to the low number of therapy naïve patients. In addition, the precise cut-off value for significant delta changes of QMG and MG-ADL score remains unclear and need to be explored in larger, longitudinal studies.

There are several limitations to our study. Although we included a rather high number of MG-patients in the cross-sectional design, our findings are limited to the rather small sample size in subgroups as well as the low number of included patients for the follow up assessment, which was due to the explorative design of our study. Future conformational and larger prospective studies are strongly needed to further examine the potential utility of CLP as a disease activity biomarker in MG.,. In addition, the main proportion of included patients was not therapy naïve as being heterogeneous in regards to disease duration and clinical severity. Nevertheless, this diversity reflects the typical demography of a specialized MG clinic. The control population was rather small, although in line with studies examining CLP in other autoimmune diseases.

## 5. Conclusion

In conclusion, this explorative cross-sectional and prospective study demonstrates that CLP levels were significantly higher in MG compared to controls. Additionally, we provide evidence, that CLP might reflect disease severity. There is an unmet need of a validated, non-invasive biomarker to assess disease activity and potentially guiding treatment. Further multicentric, longitudinal investigations are strongly needed to determine the potential utility of CLP as a biomarker for better care of patients with MG.

## Study funding

## Author contributions

Frauke Stascheit, MD: Design and conceptualized study, analyzed and interpreted the data, drafted the manuscript for intellectual content; the author takes full responsibility to the integrity of the data analyzed,
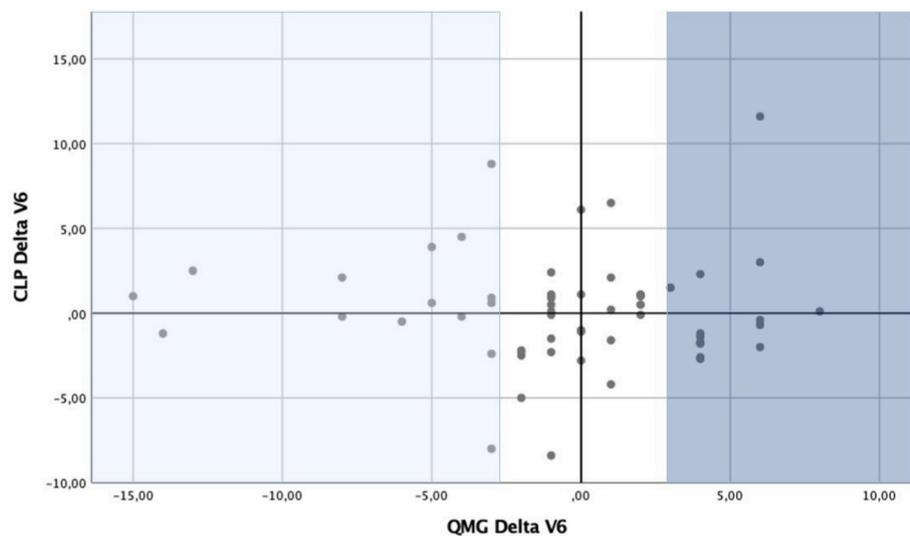
**Fig. 3.** Correlation of QMG score with CLP levels over time. Using grouped scattered plot we examined correlation in individual change (delta score) between clinical outcome parameter QMG score and CLP over two time points (baseline (n = 251 patients; year 3 (n = 58 patients). A significant change in delta QMG was defined as improvement ≥2 points presented as a positive delta (dark blue); significant change in CLP level as a negative delta (light blue).

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**Declaration of competing interest**

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