Faces and fatigue in myasthenia gravis

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Chapter 5

Correlation of C-reactive protein with severe fatigue in myasthenia gravis

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Abstract

Background and Objectives

The majority of myasthenia gravis (MG) patients suffer from fatigue, which can be defined as a subjective lack of energy and difficulty in initiating or sustaining voluntary activities. This is conceptually different from muscle weakness or muscle fatigability. Fatigue is one of the most reported symptoms in MG and has been hypothesized to be an innate mechanism to minimize muscle activity in order to protect muscles from (further) damage. The exact pathophysiology of fatigue remains unclear and it is very likely a multifactorial phenomenon. The aim of this study was to provide a better understanding on the pathophysiology of fatigue in MG.

Methods

We analyzed 38 serum biomarkers including various cytokines and myokines in a cohort of 116 anti-AChR positive MG patients. A multivariate linear regression analysis for each biomarker was performed in search for a correlation with fatigue. The following pre-selected covariates were included in the primary analysis: gender, age, disease severity, depression and anxiety scores, non-steroid immune suppressive medication and cumulative prednisone dosage in the past six months.

Results

Severe fatigue was present in 64% of patients. Results show a robust correlation between fatigue and C-reactive protein (CRP) in the primary analysis. This correlation persisted when additionally adjusting for BMI, strenuous physical activities and hemoglobulin levels.

Conclusions

Our findings suggest that chronic low grade inflammation, mediated by CRP, contributes to the pathogenesis of fatigue in MG. This aligns with the hypothesis that local peripheral inflammatory processes induce systemic inflammatory cascades responsible for fatigue.

Background

Autoimmune myasthenia gravis (MG) is a rare condition with antibodies targeted against key proteins of the neuromuscular junction (NMJ) of skeletal muscles[1]. Several types of antibodies have been identified, but the majority (approximately 80%) of patients have detectable antibodies against the acetylcholine receptor (AChR)[2]. Three mechanisms can contribute to the pathogenicity of AChR antibodies: 1) they block the binding of acetylcholine to AChRs, 2) they accelerate degradation of AChRs by crosslinking and internalization of the receptors or/and 3) they activate the complement system[2]. The predominant clinical phenotype of MG consists of fluctuating and fatigable weakness of skeletal muscles. Extraocular muscles are most frequently involved, leading to asymmetric and fluctuating ptosis and diplopia[1]. Although MG is considered to affect only NMJ, the majority of patients suffer from cognitive or mental fatigue as well[3, 4]. This type of fatigue is conceptually different from muscle weakness or fatigability and is often referred to as central fatigue, as it originates in the central nervous system[5]. Central fatigue can be best defined as a subjective lack of energy and difficulty in initiating or sustaining voluntary activities[5]. It is hypothesized that in neuromuscular diseases, central fatigue is a mechanism to minimize activities, in order to protect the muscles from further damage[5,6]. Disease-related muscle weakness may require higher levels of effort, which may contribute to central fatigue. Another hypothesis is that local peripheral inflammatory processes in auto-immune diseases may trigger systemic inflammatory cascades responsible for fatigue[6]. It has been proposed that inflammation reduces both internally and externally driven motivation states, to provide the body the opportunity to heal[7]. Several inflammatory biomarkers have previously been associated with central fatigue in numerous chronic auto-immune diseases, including rheumatoid arthritis (RA), Sjogren's disease and inflammatory bowel disease (IBD)[8]. Previous research has shown that in MG, fatigue is correlated with female gender, depressive symptoms, disease severity, body mass index (BMI) and physical activities[3, 9-12]. These data suggest that the pathophysiology of fatigue is multifactorial, although there is no evidence on causality.

Our aim was to gain a better understanding on the pathophysiology of fatigue in MG, by searching for one or more circulating serum biomarkers related to fatigue. Our second aim was to find longitudinal correlations between fatigue and potentially newly identified biomarkers.

Methods

This study was conducted between May 2022 and April 2023 at the Department of Neurology of the Leiden University Medical Center (LUMC), Leiden, the Netherlands.

Subjects

Anti-AChR positive MG patients (≥18 years) were recruited from the Dutch – Belgian Myasthenia Patient Registry[13]. Diagnosis of MG was based on clinical signs and symptoms characteristic for MG and a documented positive serological test for AChR. Exclusion criteria were: 1) a medical history of other active autoimmune disorders, with "active" being defined as having clinical symptoms or currently receiving immune modulating therapy and 2) a medical history of a neoplasm or receiving cytostatic treatment within the last year. If participants met the in- and exclusion criteria, they were scheduled in consecutive order of registration until the predetermined sample size (n = 116) was reached.

Procedures

The study consisted of two visits: one extensive baseline visit between May and August 2022 and a second shorter visit between March and April 2023. To limit the burden of study participation, the second visit was optional. After written informed consent, baseline characteristics (for more details see table 1) were collected and a baseline quantitative MG (QMG) test was performed by the researcher (A.R.) to document disease severity. Participants were asked to refrain from taking their morning pyridostigmine dosage until after the QMG test.

- ***Questionnaires at baseline and second visit***

Participants were asked to complete the following set of questionnaires on paper (baseline) or online (second visit): a) Checklist Individual Strength for fatigue (CIS-f) to assess central fatigue[14]. This is an 8-item self-assessment on experienced fatigue during the past two weeks and is scored on a 7-point Likert scale. It ranges from 8 to 56 points, with ≥35 indicating severe or clinically relevant fatigue. b) Hospital Anxiety and Depression Scale (HADS) for depressive symptoms[15] and c) MG-Activities of Daily Living (MG-ADL) for disease severity[16]. Consequently, participants were asked to provide an overview of their medication use over the past six months and information on their level of physical activities and height and weight.

- ***Baseline blood samples***

All blood samples were collected during the study visit between 10.30AM and 01.00PM to restrict potential circadian influences on biomarker results. Routine hematology (hemoglobin

(Hb), erythrocytes, thrombocytes, monocytes, lymphocytes, leukocytes + differentiation) and chemistry (C-reactive protein (CRP) and creatinine), together with components of the complement system (C1q, C3 and C4) and complement activation (classic pathway (CP) and alternative pathway (AP)) (directly stored on ice) were analyzed instantly by the clinical chemistry laboratory at the LUMC following standard operating procedures (eTable 1). All other biomarkers were analyzed by batch after collection of all samples, stored at -80°C. Serum AChR antibody titers and immunoglobulin G (IgG) were measured by the clinical chemistry laboratory at the LUMC using a radioimmunoprecipitation assay in accordance with standard operating procedures (for details see eTable 1).

- ***Serum protein measurements***

The following serum proteins-levels were determined with multiplex bead assays at the infectious disease laboratory at the LUMC[17] using Milliplex reagents (Merck, Amsterdam, The Netherlands). The assay makes use of a window of detection with a lower detection limit of <1pg/ml and the upper detection limit > 20000 pg/ml.
Brain-derived neurotrophic factor (BDNF), irisin, myostatin, fibroblast growth factor (FGF)21 were tested as part of the myokine panel (HMYOMAG-56K Human Myokine panel). Interferon γ (IFN-γ), interferon gamma-induced protein 10 (IP10), tumor necrosis factor α (TNF-α), fibroblast growth factor (FGF)2, interleukin (IL-) 1ß, 4, 6, 8, 10, 13, 15, 17a, 18 and 22 were selected as a custom made panel from the cytokine/chemokine/growth factor panel A (HCYTA-60K). Insulin-like growth factor 1 (IGF-1) and osteopontin were tested as single analytes (HIGFMAG-52K and HBNMAG-51K respectively). Assays were performed according to manufacturer's instructions with manufacturers specific standards and QC control samples. All samples were thawed and diluted in sample diluent according to manufacturer's instruction and run as single measurement. Samples were acquired on a Bio-plex 200 system and analyzed with Bio-plex Manager software v6.2.

- ***Blood samples at second visit***

A repeated sample of CRP was taken under similar circumstances 8.6 ±0.9 months later, between March and April 2023. The decision to only acquire repeated blood samples for CRP serum levels was made after the results of the primary multivariate regression analysis were available.

Statistical analyses

The sample size was based on an assumed correlation coefficient of 0.27 between a biomarker and central fatigue using a linear regression model with a significance of $p<0.05$, a power of 80% and a drop out rate of 10%.
Statistical analyses were performed using IBM SPSS Statistics version 25.0. Group data are

described by mean and standard deviation (±SD) for normally distributed data or median and interquartile range [IQR] for non-parametric data. Unpaired T-tests or Mann-Whitney U tests were used for comparison between two groups, one-way ANOVA for >2 groups. Pearson's *r* were used for bivariate correlations. A multivariate linear regression analysis was performed for each biomarker individually. If a biomarker was not detectable in 20 or more patients, it was not used for further statistical analyses. Data transformation for skewed biomarker data was not applied. Imputation for missing data was not applied. The pre-selected covariates as predictors for the regression analysis were as follows: gender, age, QMG, HADS-d, use of non-steroid immune suppressive medication yes/no and cumulative prednisone dosage in the past six months. This pre-selection was based on either a previously shown association or a supposed predictive or explanatory confounding effect on fatigue or biomarkers[3]. Significance was accepted at $p < 0.05$. Post hoc analyses were performed only on biomarkers of interest. Correction for multiple testing was only applied in post hoc analyses because of the exploratory design of the first part of the study. Biomarkers with values below calibration lines with the multiplex bead assay, were identified as undetectable with the current assay and set to zero for statistics.

Declarations

Ethics approval and consent to participate

The ethics committee of the LUMC approved the study protocol (NL72266.058.20). Written consent was obtained from all participants according to the declaration of Helsinki (2013) and in accordance with the Dutch Medical Research Involving Human Subjects Act (WMO), Good Clinical Practice (GCP) guidelines and General Data Protection Regulation (GDPR).

Results

Baseline characteristics

A study invitation was sent to 556 AChR positive MG patients. A total of 116 patients were included in consecutive order until the predetermined sample size was reached (Figure 1).

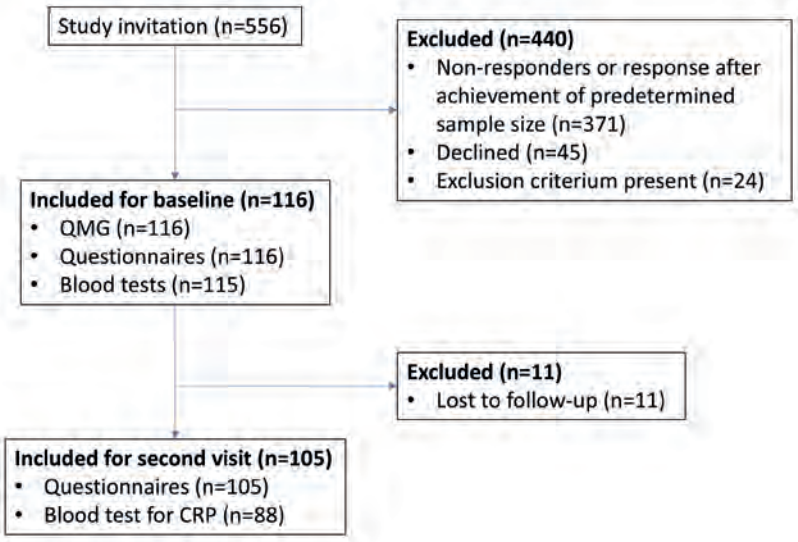

Figure 1. Study flow chart

Table 1 shows the clinical characteristics of the study population. The mean ±SD fatigue score (CIS-f) was 36.7 ±13.6 points. Severe fatigue (≥35 points) was present in 64% of patients. There was no difference in fatigue scores between males and females: 36.1 ±14.2 vs 37.3 ±13.0, p=0.65. Fatigue scores were positively correlated with disease severity: QMG: *r* 0.39 (p <0.001); R^2 0.15 and MG-ADL: *r* 0.55 (p <0.001); R^2 0.30. Figure 2 shows the median [IQR] fatigue scores and the percentage of patients with severe fatigue per MGFA (MG Foundation of America) class (score ≥35). There were no differences in fatigue scores between patients without MG medication, pyridostigmine monotherapy or steroids and/ or non-steroid immune suppressive medication (p = 0.21). Ten percent of all patients were in remission, as defined in the 2016 international consensus guidance[18], of whom 75% were in pharmacological remission.

Mean fatigue scores were significantly higher in patients with concomitant hypo- or hyperthyroid disease (n=10): 45.3 ±7.8 compared to 35.9 ±13.8 points in patients without thyroid disease (p=0.004). There was a positive correlation between BMI and fatigue scores:

Table 1. Clinical characteristics

Characteristic	Participants
Included, n (% female)	116 (48.3%)
Age, mean ±SD	62.7 ±13.0
Disease duration years, mean ±SD	11.6 ±10.6
QMG score, mean ±SD	6.2 ±5.0
MG-ADL score, mean ±SD	4.1 ±3.4
MGFA class, n (%)	.
· Remission	12 (10.3%)
· I	6 (5.2%)
· II	58 (50%)
· III	37 (31.9%)
· IV	3 (2.6%)
· V	0
Fatigue score (CIS-f), mean ±SD	36.7 ±13.6
Severe fatigue (CIS-f ≥35), %	63.8%
Depression score (HADS-d), mean ±SD	4.3 ±3.6
BMI, mean ±SD	27.6 ±5.3
Strenuous activities, n (%)	.
· None	86 (74.8%)
· Once a week	8 (7.0%)
· Twice a week	14 (12.2%)
· More than twice a week	7 (6.1%)
MG disease specific treatment, n (%)	
· No disease specific treatment	15 (12.9%)
· Pyridostigmine	67 (57.8%)
- Pyridostigmine monotherapy	24 (20.7%)
· Prednisolone	48 (41.4%)
- Prednisolone monotherapy[a]	19 (16.4%)
· Non-steroid immune suppressive medication	58 (50%)
· IV immune globulins	12 (10.3%)
· Plasmapheresis	1 (0.9%)
Non-steroid immune suppressive medication	
· Azathioprine	30 (25.9%)
· Mycophenolate mofetil	18 (15.5%)
· Cyclosporine	5 (4.3%)
· Methotrexate	4 (3.4%)
· Efgartigimod	3 (2.6%)
· Tacrolimus	2 (1.7%)

Abbreviations: [a] = with or without pyridostigmine; BMI = body mass index; CIS-f = checklist individual strength – fatigue; HADS-d = hospital anxiety depression scale – depression sub score; IV = intravenous; MG-ADL = myasthenia gravis – activities of daily living; MGFA = MG foundation of America; QMG = quantitative myasthenia gravis

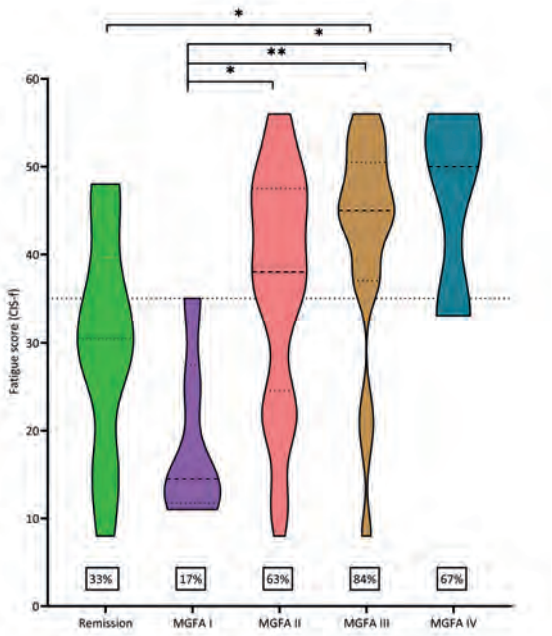

Figure 2. Median fatigue scores (CIS-f) per MGFA class. Horizontal line indicates cut-off for clinically relevant fatigue (≥35), boxes at bottom of figure shows percentage clinically relevant fatigue per MGFA class. Abbrevations: CIS-f = Checklist Individual Strenght – fatigue; *p<0.01; **p<0.001

r 0.23, p=0.015; R^2 0.05. Mean BMI scores did not differ between patients with or without steroids (currently or in the past six months): 27.6 ±4.7 versus 27.6 ±5.7 (p=0.99). A majority of patients (n=86, 74%) did not engage in strenuous physical activities, such as running, cycling, or engaging in sports. The mean fatigue score of patients who did not perform strenuous physical activities was 39.5 ±12.2, compared to 28.1 ±14.4 (p <0.001) in patients performing regular activities (≥ once a week). More than half of patients consumed ≥1 unit of alcohol a week, 31% reported never drinking alcohol and 14% less than one consumption a week. The mean fatigue score did not differ between patients who never consumed alcohol and those who consumed ≥1 unit a week (38.3 ±13.9 vs 35.4 ±14.0, p=0.32). A previous COVID infection was reported by 53% of patients. The mean fatigue score of patients with a previous COVID infection did not differ from patients without: 36.8 ±14.2 vs 36.1 ±13.1, p=0.77.

eTable 2 shows the results of a multivariate linear regression analysis for fatigue, adjusted for pre-selected baseline covariates (gender, age, QMG, HADS-d, use of non-steroid immune suppressive medication and cumulative prednisone dosage). Fatigue was positively correlated with the QMG score (ß 0.58, p=0.009) and the HADS-d (ß 1.99, p <0.001).

Fatigue and biomarkers at baseline

Table 2 shows mean serum concentrations of all investigated potential biomarkers. Separate multivariate linear regression analyses were performed to calculate adjusted correlations between fatigue and individual biomarkers (table 2). This analysis was repeated for each biomarker. After adjusting for the pre-selected baseline covariates a positive correlation with fatigue was found for CRP (ß 0.78, p=0.002). Fatigue was negatively correlated with Hb (ß -2.73, p=0.035) and basophilic granulocytes (ß -107.56, p=0.045).

Bivariate (unadjusted) analyses were performed for those biomarkers from the multivariate analysis that correlated with fatigue. This resulted in a negative correlation for fatigue with Hb (*r* -0.19, p=0.038; R^2 0.04). Anemia (Hb <7.5 (female) or <8.5 (male) mmol/l) was present in 17% of patients. The mean fatigue score in patients with anemia was 44.2 ±7.1 compared to 35.1 ±14.1 in patient without anemia (p <0.001). No bivariate correlation between fatigue and CRP (*r* 0.18, p=0.051) or basophilic granulocytes (*r* -0.12, p=0.20) was found. The major stimulus for CRP production is IL-6. A positive correlation was found between CRP and IL-6 (*r* 0.36, p=0.015; R^2 0.13), but no correlation was found between fatigue and IL-6 (*r* -0.10, p=0.49).

Post hoc analyses were only performed for CRP. With a post hoc Bonferroni correction for multiple testing, significance was accepted at p <0.0125. The positive correlation with fatigue persisted when additionally adjusting for BMI (ß 0.68, p=0.01), strenuous activities (ß 0.80, p=0.001) or Hb (ß 0.68, p=0.007). Adjusting for all post hoc variates simultaneously in the primary model also showed a positive correlation between CRP and fatigue (ß 0.60, p=0.021). CRP was not correlated with QMG score (*r* 0.06, p=0.56) or MG-ADL (*r* -0.02, p=0.84).

Longitudinal results

The majority (n=105, 91%) of patients participated in taking measurements at the second time point (figure 1), of whom 17 patients only completed the online questionnaires, resulting in a drop-out rate of 24% for blood withdrawals at the second time point. The mean interval between baseline and second visit was 8.6 ±0.9 months. The mean fatigue score at the second visit was 36.3 ±12.2 points, a decrease of 0.4 points compared to baseline.

Figure 3 shows the dichotomized fatigue results between baseline and the second visit. Of the patients with severe fatigue (CIS-f ≥35) at baseline, 18% had a decrease in fatigue scores below the cut-off for severe fatigue (CIS-f <35) and were now categorized as not severely fatigued. A total of 38% of patients without severe fatigue at baseline, had an increase of fatigue above the cut-off and were now categorized as severely fatigued.

Figure 4 shows the change (delta) between baseline and the second visit of MG-ADL and CRP plotted against the change in CIS-f scores. Compared to baseline, mean MG-ADL scores improved 0.5 points to 3.6 ±3.2 points. Median CRP at the second visit was 1.3 [0.7-2.3] mg/L, a decrease of 0.1 mg/L. There was a positive correlation between delta CIS-f and delta MG-ADL (*r* 0.40, p <0.001; R^2 0.16) but no correlation between delta CIS-f and delta CRP (*r* -0.02, p=0.88).

To replicate the observed correlation between fatigue and CRP at baseline, a repeated multivariate linear regression analysis was performed with data collected at the second visit, with adjustment for the same covariates as those used for the first analysis (n=88). The MG-ADL was used for disease severity. The observed correlation between fatigue and CRP at baseline was not found at the second visit (ß 0.37, p=0.20).

Next, to study the predictive potential of CRP for fatigue, a multivariate linear regression analysis was performed with CIS-f at the second visit as dependent covariate, together with the pre-selected baseline covariates (n=104). There was no correlation between CRP at baseline and fatigue at the second visit (ß 0.15, p=0.55). Additionally, fatigue at baseline was not correlated with CRP at the second visit (n=88, ß 0.06, p=0.93).

Table 2. Mean serum concentrations of all potential biomarker at baseline and results of multivariate linear regression analyses (statistically significant results in bold)

Biomarker	Result (mean ±SD / median [IQR])	Reference range and unit	Detectable in, n (%)	ß	SE	95% CI	Sig.
Hemoglobin	8.7 ±0.8	7.5 – 10[a] / 8.5 – 11[b] mmol/L	116 (100%)	-2.73	1.30	-5.3 – -0.19	0.035
Erythrocytes	4.6 ±0.4	4.0 – 5.0[a] / 4.5 – 5.5[b] x10*12/L	116 (100%)	-3.87	2.31	-8.40 – 0.67	0.10
Thrombocytes	243 ±65	150 – 400 x10*9/L	116 (100%)	0.01	0.02	-0.02 – 0.05	0.42
Monocytes	0.5 ±0.2	0.1 – 1.0 x10*9/L	116 (100%)	4.76	5.47	-5.97 – 15.48	0.39
Lymphocytes	1.3 ±0.7	1.0 – 3.5 x10*9/L	116 (100%)	-1.23	1.54	-4.25 – 1.80	0.43
Leukocytes	7.0 ±2.1	4.0 – 10.0 x10*9/L	116 (100%)	-0.34	0.55	-1.42 – 0.74	0.54
Neutrophil gran.	5.0 ±2.0	1.5 – 7.5 x10*9/L	116 (100%)	-0.26	0.65	-1.53 – 1.02	0.69
Eosinophil gran.	0.1 ±0.1	<0.5 x10*9/L	116 (100%)	-12.51	10.90	-33.87 – 8.85	0.25
Basophil gran.	0.04 ±0.02	<0.2 x10*9/L	116 (100%)	**-107.56**	**53.74**	**-212.88 – -2.24**	**0.045**
CRP	1.4 [0.7 – 3.2]	<0.5 mg/L	115 (100%)	**0.78**	**0.25**	**0.29 – 1.28**	**0.002**
Creatinine	76 ±17.8	49 – 90 µmol/L	116 (100%)	0.08	0.07	-0.06 – 0.22	0.25
C1q	118.0 ±17.7	102 – 171 mg/L	115 (100%)	0.06	0.06	-0.1 – 0.2	0.30
C3	1.1 ±0.2	0.9 – 2 g/L	115 (100%)	7.06	5.05	-2.8 – 16.9	0.16
C4	239.8 ±69.9	95 – 415 mg/L	115 (100%)	0.00	0.02	-0.03 – 0.03	0.77
AP	74.2 ±20.0	>39%	115 (100%)	0.07	0.05	-0.04 – 0.17	0.20
CP	107.3 ±20.4	>74%	115 (100%)	0.01	0.05	-0.09 – 0.11	0.85
Anti-AChR	15.0 [1.9 – 42.0]	0.0 – 0.5 nmol/L	114 (100%)	-0.01	0.01	-0.04 – 0.01	0.32
Total IgG	10.6 ±3.6	7 – 16 g/L	114 (100%)	-0.24	0.30	-0.82 – 0.34	0.41
IL1b	0.0 [0.0 – 0.0]	pg/ml*	10 (8.8%)	NP			
IL4	0.0 [0.0 – 0.0]	pg/ml*	20 (13.9%)	-0.10	0.13	-0.35 – 0.15	0.43
IL6	0.0 [0.0 – 1.5]	pg/ml*	46 (40.4%)	-0.22	0.27	-0.75 – 0.31	0.42
IL8	3.3 [2.2 – 5.2]	pg/ml*	113 (99.1%)	-0.01	0.02	-0.06 – 0.04	0.66
IL10	0.0 [0.0 – 4.0]	pg/ml*	40 (35.1%)	-0.13	0.11	-0.34 – 0.08	0.22
IL13	0.0 [0.0 – 0.0]	pg/ml*	17 (14.9%)	NP			
IL15	0.0 [0.0 – 0.0]	pg/ml*	13 (11.4%)	NP			
IL17a	0.0 [0.0 – 0.0]	pg/ml*	6 (5.3%)	NP			
IL18	0.0 [0.0 – 0.0]	pg/ml*	27 (23.7%)	-0.02	0.05	-0.13 – 0.08	0.67
IL22	0.0 [0.0 – 0.0]	pg/ml*	17 (14.9%)	NP			
TNF-α	0.0 [0.0 -13.0]	pg/ml*	56 (49.1%)	-0.01	0.01	-0.03 – 0.01	0.37

IGF-1	0.0 [0.0 – 0.0]	pg/ml*	25 (21.9%)	0.00	0.00	-0.00 – 0.00	0.85
FGF-2	0.0 [0.0 – 0.0]	pg/ml*	20 (13.9%)	-0.00	0.00	-0.01 – 0.01	0.53
FGF-21	0.0 [0.0 – 0.0]	pg/ml*	15 (13.2%)	NP			
Osteopontin	15.3 ±13.0	ng/ml*	114 (100%)	0.00	0.00	-0.00 – 0.00	0.74
Myostatin	0.0 [0.0 – 0.0]	pg/ml*	10 (8.8%)	NP			
Irisin	0.0 [0.0 – 0.0]	pg/ml*	15 (13.2%)	NP			
IFNγ	0.0 [0.0 – 0.0]	pg/ml*	20 (13.9%)	0.02	0.08	-0.16 – 0.31	0.83
IP10	269.7 ±149.9	pg/ml*	113 (99.1%)	0.01	0.01	-0.01 – 0.02	0.41
BDNF	11.9 ±4.4	ng/ml*	114 (100%)	0.00	0.00	-0.00 – 0.00	0.53

Abbreviations: aFemale reference range; bmale reference range; * = standardized reference range not available; AChR = acetylcholine receptor; BDNF = brain derived neurotrophic factor; CRP = C-reactive protein; FGF = fibroblast growth factor; IFN-γ = interferon γ; IGF-1 = insulin-like growth factor 1; IL = interleukin; IP10 = IFN-γ-induced protein 10; NP = not performed; TNF-α = tumor necrosis factor α;

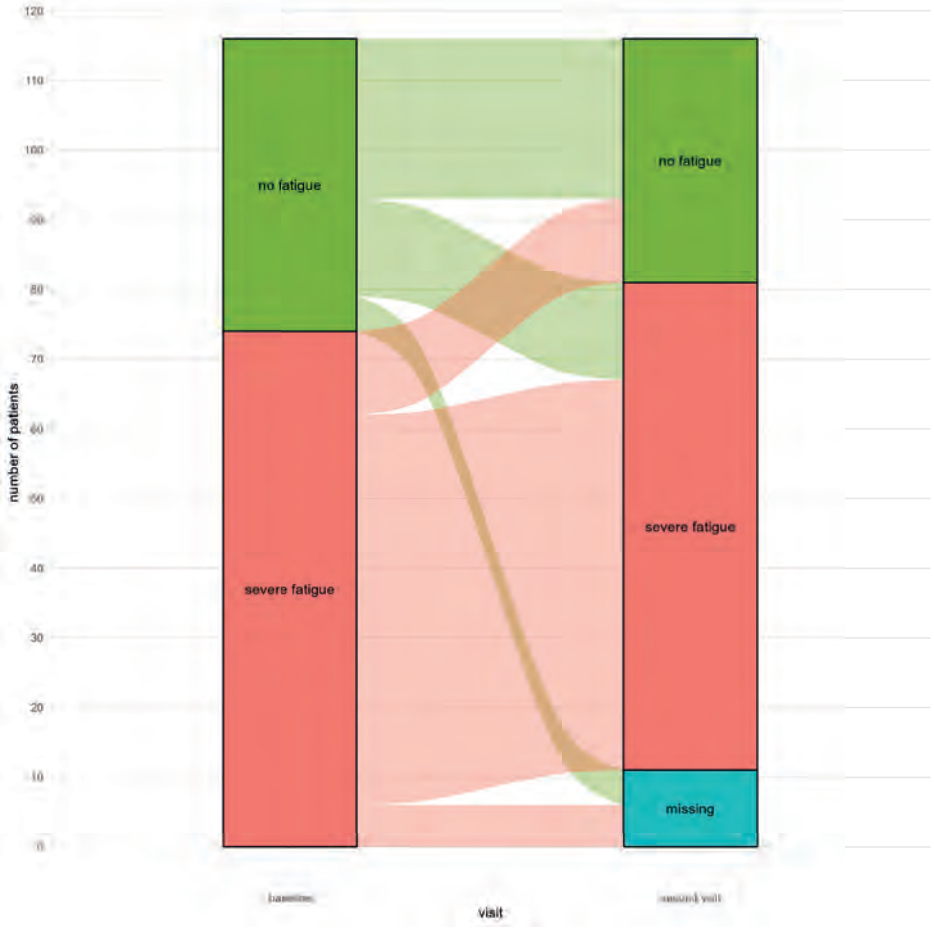

Figure 3. Switch from no severe or severe fatigue from baseline to the second visit.

Discussion

This study investigated circulating serum biomarkers for fatigue in MG. The results showed a robust correlation between fatigue and CRP at the first study visit. This correlation is independent of several covariates, including disease severity, cumulative prednisone dosage and Hb levels. This finding is in accordance with results found in other autoimmune diseases, such as rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) and has also been observed in large, general population cohort studies[19-23]. This points towards a common, rather than an MG-specific fatigue pathway, possibly implicating low-grade inflammation as a cause of fatigue. However, the prevalence of fatigue in MG is much higher than in the

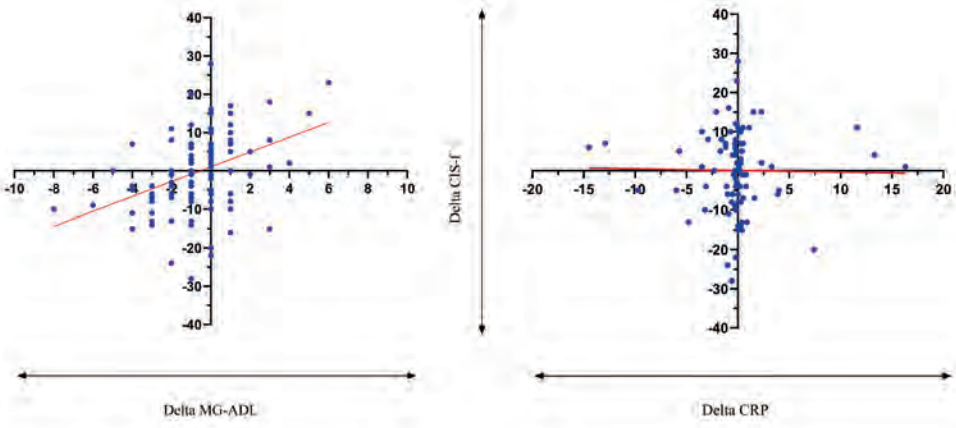

Figure 4. Change over time (delta) of disease severity (MG-ADL), fatigue (CIS-f) and C-reactive protein (CRP).

general population[24], suggesting that the threshold to develop fatigue is lower or that the exposure to fatigue-inducing triggers is higher in MG patients due to other, currently unknown contributing factors. Remarkably, increased CRP serum levels are not a typical feature in MG, unlike RA and IBD[25, 26]. This is supported by the absence of an association between CRP and clinical MG disease severity, as measured with QMG and MG-ADL. Indeed, CRP levels were within the normal range in most patients, and the median level for the entire study population was only slightly elevated: 1.4 mg/l [0.7 – 3.2] (reference <0.5 mg/l). Strikingly, even within these relatively low ranges, CRP was correlated with fatigue, suggesting low grade inflammation as part of the pathogenesis of fatigue in MG.

In accordance with previous research[3], fatigue is also strongly correlated with disease severity and depressive symptoms. Age and the female gender were not correlated with fatigue in the current study, most likely due to the smaller sample size[3]. When looking at the most frequently used immune suppressive therapies, there was no correlation of fatigue with the cumulative prednisone dosage over the past six months or the use of azathioprine.

CRP, IL-6 and fatigue

CRP is an acute phase pentameric protein, produced by hepatocytes in the liver[27]. Its production is mainly stimulated by IL-6 and, to a lesser extent by TNF-α and IL-1ß. IL-6 possesses both pro- and anti-inflammatory characteristics as a cytokine and a muscle produced myokine[28], and may play a key role in the pathogenesis of anti-AChR MG, but the exact mechanism is not yet fully understood[29-32].

Remarkably, despite the correlation with CRP, we did not find a correlation between IL-6 and fatigue. This could be due to the fact that IL-6 levels were below the detection threshold of the multiplex bead assay in 60% of patients, but these low levels may have been sufficient enough to stimulate CRP production. Another explanation could be the relatively small sample size or as a result of the high prevalence of immune suppressive therapy in the study population, which reduces serum IL-6 levels[31]. Finally, we did not measure the soluble IL-6 receptor (sIL-6R), which binds IL-6[34].

A previous study in a large sample of the working British population showed a correlation between fatigue, CRP and IL-6[21]. Furthermore, in RA, IL-6 inhibitors have shown to improve fatigue[21, 35, 36].

Immune-to-brain communication pathways

The exact mechanism by which peripheral circulating CRP or other cytokines contribute to central fatigue remains unclear. Central fatigue is a behavioral pattern that arises in the brain[5, 6], and several immune-to-brain communication pathways exist through which peripheral circulating proinflammatory cytokines communicate with the brain[7, 37, 38]. First, local cytokines can activate afferent nerves at the site of inflammation, for example the vagal or trigeminal nerve. A second route is the humoral pathway, in which pathogen-associated molecular patterns elicit pro-inflammatory cytokine production in the circumventricular organs[7, 37]. These cytokines can diffuse across the blood-brain barrier (BBB). A third pathway involves activation of IL-1 receptors in brain venules by circulating cytokines, causing local production of prostaglandin E2[7, 37]. A fourth pathway comprehends circulating cytokines gaining access across the BBB through saturable transport systems[7, 37]. There is also evidence that an increase in proinflammatory cytokines triggers a disruption of the BBB[38]. Through these pathways, activation of microglia cells takes place, resulting in the release of brain inflammatory cytokines and prostaglandins. These mediators may be responsible for the development of several behavioral responses, including fatigue[7].

Other biomarkers and fatigue

Fatigue is a well-known symptom in patients with anemia[41], therefore the observed negative correlation between fatigue and Hb levels was not unexpected. The exact mechanism of fatigue is unclear but could be related to lower oxygenation of body tissues[41]. There is no clear explanation for the negative correlation between basophilic granulocytes and fatigue. Basophilic granulocytes are white blood cells involved in allergic reactions and parasitic infections[42]. Previous research studying the association between white blood cell counts and fatigue in a large sample of the general population did not find any correlation between basophilic granulocytes and fatigue[43].

Except for Hb and basophilic granulocytes, we did not find any correlation between fatigue and the other studied biomarkers, including MG-specific AChR antibodies or the complement system. A recent randomized controlled trial in MG with zilucoplan, a complement inhibitor, demonstrated a significant reduction in fatigue scores in patients treated with zilocoplan compared to placebo[44]. However, these results were not adjusted for the improvement in disease severity due to zilucoplan and therefore this study does not provide information on the role of complement in the specific pathogenesis of fatigue.

Predictive value of CRP

Previous research demonstrated that CRP was a prospective and predictive biomarker for fatigue six months later in post stroke fatigue and three or five years later in the general population[21, 22, 49]. We could not reproduce a similar predictive value of CRP in MG patients, possibly due to our relatively small sample size (n=104) compared to these prior studies.

Limitations

The goal of this study was to search for a potential fatigue-biomarker to increase our understanding on the pathogenesis of fatigue specific to MG. Therefore, non-fatigued MG patients served as a control group. The lack of a healthy control group could be considered a limitation. Secondly, for some biomarkers the sensitivity of the assay was limited, which resulted in undetectable values. For IL-6 there was no difference in fatigue scores below or above the detection range of IL-6. Therefore, no additional (more sensitive) IL-6 assay was performed. The primary multiple linear regression analysis was repeated for each individual biomarker, we did not correct for multiple testing due to the exploratory nature of this experiment. With a post hoc Bonferroni correction ($p < 0.00167$), no biomarkers would reach significancy. Nonetheless, the main finding of this paper, the correlation between serum CRP concentrations and fatigue, remained significant after correcting for multiple testing. Although the results of the baseline visit are robust, we could not replicate this finding in a set of sera collected at a second visit. The number of participants at this second time point was lower than the predetermined sample size, due to a much higher drop-out rate of 24% than the predicted 10% as a result of the optional nature of the second visit. Due to this drop-out rate, the second measurement lacked statistical power and this is the most likely cause of the observed lack of correlation between fatigue and CRP. Furthermore, closer examination of the dataset at the second time point revealed two apparent outliers with very high CRP scores and low fatigue scores. Exclusion of these two subjects would have led to a significant correlation. Finally, CRP serum levels are affected by seasonal influences[50]. Data for the baseline visit was collected in summer, while data for the second visit was collected in March and April. Although median CRP serum levels did not differ between the two datasets, individual measurements may have been affected by seasonal changes.

Conclusion

The results of this study suggest that ongoing chronic low grade inflammation is part of the pathogenesis of fatigue in MG. This is supported by the results of previous research on fatigue both in autoimmune disorders and in the general population. However, low grade inflammation is probably only a partial contributor to fatigue and a multifactorial pathogenesis remains likely.

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Supplemental files

eTable 1. Standard operating laboratory procedures

Biomarker(s)	Method
Routine hematology	XN9000 analyser (Sysmex): colorimetry and flow cytometry
CRP	Roche COBAS 8000 Modular - c702: turbidimetry, antigen – antibody complex
Creatinine	Roche Cobas C8000 Modular - c702: enzymatic method, photometry wavelength 546/700 nm
Complement C1q	Siemens Prospec: nephelometry, antigen-antibody complex
Complement C3 and C5	Optilite (The Binding Site): turbidimetry
Complement activation routes	ELISA (Wieslab)
AChR antibodies	Radio immune assay (RSR)
Total IgG	Cobas 8000, C502 module: turbidimetry, antigen – antibody complex
Brain-derived neurotrophic factor (BDNF), osteopontin, irisin, myostatin, insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF) 2,21, interferon γ (IFN-γ), interferon gamma-induced protein 10 (IP10), tumor necrosis factor α (TNF-α), interleukin (IL) 1ß,4,6,8,10,13,15,17a,18,22	Myokine panel, Human Bone Panel, Human IGF-I,II endocrine assay and the Human Cytokine/ Chemokine Immunology panel, Milliplex, Merck. Samples were acquired on a BioPlex 200 system and analyzed with BioPlex manager software (v6.2). Briefly, sera were diluted as suggested by the manufacturer for the different analytes and incubated with the specific antibody coated beads followed by biotin labeled detection antibodies and streptavidin-PE for quantification

eTable 2. Multivariate linear regression analysis of factors potentially associated with fatigue.

Dependent variable	CIS-f score			
Predictor	ß	SE	95% CI	Sig.
Female gender	1.16	2.35	-3.45 – 5.77	0.62
Age	-0.13	0.08	-0.29 – 0.03	0.12
QMG	0.58	0.22	0.14 – 1.02	0.009
HADS-d	1.99	0.30	1.38 – 2.58	<0.001
Non-steroid immune suppressive medication	0.84	2.01	-3.09 – 4.78	0.67
Cumulative prednisone dosage	0.00	0.00	-0.00 – 0.00	0.87