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Research Paper

Exploring the role of circulating glial fibrillary acidic protein and neurofilament light chain in myasthenia gravis

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ABSTRACT

Background: Myasthenia gravis (MG) is an autoimmune disorder of the neuromuscular junction caused by antibodies against the acetylcholine receptor (AChR) or muscle-specific kinase (MuSK). Although these antibodies enable diagnosis, they correlate poorly with disease activity and prognosis. Emerging data suggest that MG involves broader neuroimmune mechanisms beyond the peripheral synapse. Glial fibrillary acidic protein (GFAP), a marker of astroglial activation, and neurofilament light chain (NfL), a marker of neuroaxonal injury, have been proposed as circulating biomarkers in several neurological diseases. This study aimed to evaluate serum GFAP and NfL concentrations in MG patients and to assess its potential diagnostic utility.

Methods: In this retrospective case–control study, 137 patients with confirmed MG and 338 healthy controls were enrolled. Clinical classification followed MGFA criteria. Anti-AChR and anti-MuSK antibodies were measured by ELISA. Serum GFAP and NfL concentrations were quantified using a fully automated chemiluminescent immunoassay (Lumipulse G1200). Group comparisons were performed using non-parametric tests, correlations using Spearman analysis, and independent determinants assessed with multivariable linear regression. Receiver operating characteristic (ROC) curve analysis was performed to evaluate diagnostic performance.

Results: Serum GFAP concentrations were higher in MG patients than controls (44.6 vs 29.6 pg/mL) and strongly correlated with age ($r = 0.64$, $p < 0.0001$), but not with antibody titers. In multivariable regression, age was the main independent determinant of GFAP ($\beta = 0.00827$, $p < 0.0001$), whereas MG status was not. GFAP showed moderate diagnostic performance (AUC = 0.704). In an age- and sex-matched analysis, NfL concentrations were modestly higher in MG patients compared with controls (30.2 vs 15.5 pg/mL; $p = 0.047$). A moderate positive correlation between GFAP and NfL was observed in both groups.

Conclusions: Circulating GFAP levels are elevated in MG but are primarily driven by age and sex rather than disease-specific mechanisms, limiting their diagnostic utility. NfL shows a modest increase, suggesting subtle neuroaxonal involvement; however, its clinical relevance remains uncertain. Together, these findings indicate that GFAP and NfL reflect non-specific neurobiological processes rather than robust biomarkers of MG.

1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder of the neuromuscular junction characterized by fluctuating fatigable muscle weakness affecting ocular, bulbar, and skeletal muscles [1,2]. The disease results from pathogenic autoantibodies that target components of the postsynaptic membrane, most commonly the nicotinic acetylcholine receptor (AChR) in 80–85% of patients, or muscle-specific kinase (MuSK) in 5–8% of cases [3,4]. These autoantibodies impair neuromuscular transmission through multiple mechanisms, including

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complement-mediated destruction of the neuromuscular junction, accelerated receptor degradation, and functional blockade of acetylcholine binding [1]. With an annual incidence of 8–10 cases per million persons and a prevalence of 150–250 cases per million, MG represents the most common disorder affecting the neuromuscular junction [5].

The diagnosis of MG relies on the integration of clinical features, serological testing for disease-specific autoantibodies, and electrophysiological studies demonstrating impaired neuromuscular transmission [6]. Anti-AChR antibodies are detected in approximately 85% of patients with generalized disease and 50–60% of those with ocular disease, while anti-MuSK antibodies are found in 30–60% of AChR antibody-negative patients. Moreover, about 1–5% present antibodies against lipoprotein receptor-related protein 4 (LRP4). These antibodies serve as critical diagnostic biomarkers and define distinct disease subgroups with different clinical phenotypes and treatment responses [7]. However, approximately 10–15% of MG patients remain seronegative for both AChR and MuSK antibodies, and there is no absolute correlation between antibody titers and disease severity in individual patients, although changes in antibody levels within individual patients often correlate with clinical status [4,8]. Despite advances in MG diagnostics and therapeutics, predictive biomarkers to personalize treatment and monitor disease activity remain underdeveloped [9]. While anti-AChR and anti-MuSK antibodies confirm diagnosis and guide initial treatment decisions, their limited correlation with disease severity and treatment response highlights the need for additional biomarkers that might reflect disease activity, predict clinical outcomes, or identify patients at risk for disease exacerbations. Emerging research has explored various candidate biomarkers, including microRNAs, complement factors, and metabolites, but none have achieved widespread clinical implementation [10].

Glial fibrillary acidic protein (GFAP) is a type III intermediate filament protein that serves as the major cytoskeletal constituent of astrocytes in the central nervous system (CNS). Under physiological conditions, GFAP provides structural support and maintains the mechanical integrity of astrocytes [11]. In neurological disorders, astrocyte activation and injury lead to the release of GFAP into biofluids, where it can be detected using ultrasensitive immunoassays [12,13]. Although MG has traditionally been characterized as a disorder of the neuromuscular junction, associations between MG and CNS dysfunction have been recognized for more than eight decades. Epidemiological and clinical studies have reported an increased prevalence of psychiatric disorders, epilepsy, and multiple sclerosis among patients with MG, as well as electroencephalographic (EEG) abnormalities and altered evoked potentials [14,15]. Furthermore, accumulating evidence indicates that MG is accompanied by systemic immune activation, complement dysregulation, and elevated circulating inflammatory mediators capable of influencing CNS homeostasis and potentially promoting neuroinflammatory processes [16]. In this context, circulating GFAP may represent a peripheral biomarker of astrocytic stress secondary to chronic immune activation, even in the absence of overt primary CNS pathology. Neurofilament light chain (NfL) is a structural component of the neuronal cytoskeleton released during axonal injury and is widely recognized as a circulating biomarker of neuroaxonal damage [17–19]. The combined assessment of GFAP and NfL may therefore provide complementary information on astroglial and neuroaxonal processes potentially involved in MG.

GFAP has been extensively studied as a biomarker in several clinical conditions including traumatic brain injury, stroke, Alzheimer's disease, and multiple sclerosis [20–22]. However, to date, circulating GFAP has not been systematically investigated in MG, and its potential utility as a diagnostic or prognostic biomarker in this disease remains unknown.

The primary objective of this study was to measure serum GFAP and NfL concentrations in patients with MG compared with healthy controls and to determine whether MG status independently influences circulating biomarkers levels after accounting for demographic factors. Secondary objectives included evaluating correlations between GFAP and NfL levels and disease-specific serological markers (anti-AChR and anti-MuSK antibody titers) as well as identifying demographic and clinical determinants of these biomarkers in this population. By characterizing GFAP and NfL levels in MG and their relationship to disease-specific features, this study aims to provide foundational data regarding the potential utility of these biomarkers in MG.

2. Materials and methods

2.1. Study population

We conducted a retrospective observational case-control study at the University Hospital “P. Giaccone” in Palermo, Italy, including patients with a confirmed diagnosis of MG and healthy controls.

Expert neurologists recruited MG patients at the Unit of Neurology of the A.O.U.P. “Paolo Giaccone”, University of Palermo, Italy. The diagnosis of MG was made according to the International Consensus Guidance for the Management of MG [23]. All patients underwent a complete medical and neurological evaluation, routine blood tests including anti-AChR and anti-MuSK antibody detection, electrophysiological investigations (repetitive nerve stimulation and/or single-fiber electromyography), chest imaging to evaluate thymic abnormalities, and other standard diagnostic procedures for MG. Patients were classified according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification at disease onset and at each follow-up visit [24]. Controls were healthy individuals enrolled as volunteers at the Institute of Clinical Biochemistry, Clinical Molecular Medicine, and Clinical Laboratory Medicine, University of Palermo, Italy.

Controls did not have age or physiological deterioration of organs and systems, including deafness and visual problems.

Each participant provided informed consent. The study was conducted in compliance with the principles of the Declaration of Helsinki and was approved by the “Palermo I" Ethics Committee (approval no. 05/2021, 19 May 2021).

2.2. Biochemical analysis

All samples were processed at the Institute of Clinical Biochemistry, Clinical Molecular Medicine, and Clinical Laboratory Medicine, University of Palermo, Italy. Venous blood was collected from each participant into dry tubes and centrifuged at 1800 $\times g$ for 10 min at room temperature. The obtained serum was immediately aliquoted into 500 μL portions in 2 mL polypropylene tubes and stored at −80 °C until analysis.

Anti-AChR and anti-MuSK autoantibodies were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits: the AChR Autoantibody ELISA Kit (RSR Ltd., Cardiff, UK) and the MuSK Autoantibody ELISA Kit (IBL International GmbH, Männedorf, Germany), respectively, in accordance with the manufacturers' instructions [25,26].

Each sample was analyzed in duplicate, and final concentrations were calculated as the mean of the two measurements. Anti-AChR results were classified as negative (<0.45 nmol/L) or positive (≥0.45 nmol/L). Anti-MuSK results were categorized as negative (<0.4 U/mL) or positive (≥0.4 U/mL).

Serum GFAP and NfL concentrations were measured using the Lumipulse G GFAP assay on the fully automated Lumipulse G1200 platform (Fujirebio Inc., Tokyo, Japan), in accordance with the manufacturer's instructions.

The Lumipulse platform provides high analytical sensitivity and specificity for the detection of both GFAP and NfL. For GFAP, the assay demonstrates a limit of detection (LoD) of 1.8 pg/mL and a limit of quantification (LoQ), defined at a 10% coefficient of variation (CV), of 16.6 pg/mL with an overall assay precision of <5% CV. Full-length recombinant human GFAP protein (LSBio, Shirley, MA, USA) is used as the reference standard. The analytical measurement range extends from 4.0

to 5000.0 pg/mL.

For NfL, the manufacturer reports a LoD of 1.99 pg/mL and a LoQ of 3.25 pg/mL, with an intra-assay CV <5%. All samples, calibrators, and controls were analyzed in duplicate, and the mean value was used for statistical analysis. No sample dilution was required prior to measurement. According to the manufacturer's kit insert, the analytical measurement range assays extend from 4.0 to 5000.0 and 2.0 to 5000 pg/mL for GFAP and NfL respectively.

2.3. Statistical analysis

Statistical analyses were performed using MedCalc statistical software v. 23.4.5 (MedCalc Software Ltd., Ostend, Belgium). The normality of data distribution was assessed using the Shapiro–Wilk test. Since the variables were not normally distributed, descriptive statistics are presented as the median and interquartile range (IQR), and nonparametric statistical methods were applied. Group comparisons were conducted using a Mann-Whitney *U* test.

Age and sex matching between groups was performed using nonparametric methods [27]. Correlation between biomarkers has been evaluated with Spearman method and 0.05 significance level. Receiver operating characteristic (ROC) curve analysis was performed to assess the diagnostic performance of serum GFAP and NfL in discriminating MG patients from healthy controls. The optimal cut-off value was determined using the Youden index.

3. Results

This study included a total of 475 individuals, 137 patients with a confirmed diagnosis of MG (M: F 43.8%; median age 64 ys) and 338 controls (M: F 63:4%; median age 49ys).

Demographic, biochemical, and clinical characteristics of patients with MG are shown in Table 1.

In the overall cohort, mean GFAP levels were higher in MG patients compared with controls (44.6 pg/mL vs 29.64 pg/mL, respectively) (Fig. 1). Across the entire study population, a strong positive correlation was observed between age and GFAP levels ($r = 0.64$, $p < 0.0001$) (Fig. 2), indicating that circulating GFAP concentrations increase substantially with advancing age. No significant association was detected between group (MG vs controls) and sex (Fisher's exact test, $p = 1.000$). No significant correlation was observed between serum GFAP concentrations and anti-AChR antibody titers (Spearman $\rho = -0.004$, 95% CI −0.152 to 0.145, $p = 0.961$) or anti-MuSK antibody titers (Spearman $\rho = 0.059$, 95% CI −0.111 to 0.227, $p = 0.495$), indicating that circulating GFAP levels were independent of serological autoimmune activity.

To evaluate independent determinants of circulating GFAP, a multiple linear regression analysis was performed in the overall cohort ($n = 475$) using log-transformed GFAP as the dependent variable and including age, sex (0 = male, 1 = female), group (MG vs controls), and their interaction term as predictors. The model was statistically significant ($F = 103.97$, $p < 0.0001$) explaining 47.0% of the GFAP variability ($R^2 = 0.470$; adjusted $R^2 = 0.466$). Age emerged as the strongest independent determinant ($\beta = 0.00827$, 95% CI 0.00710–0.00944, $p < 0.0001$), corresponding to an approximate 0.83% increase in GFAP concentration per year. Female sex was also independently associated with higher GFAP concentrations ($\beta = 0.114$, 95% CI 0.079–0.149, $p < 0.0001$), corresponding to approximately 12% higher GFAP levels compared with males.

MG status was not independently associated with GFAP concentrations ($\beta = -0.124$, 95% CI −0.286 to 0.038, $p = 0.134$). However, a small but statistically significant age × group interaction was observed ($\beta = 0.00264$, 95% CI 0.00007–0.00520, $p = 0.044$), indicating a slightly steeper age-related increase of GFAP in MG patients compared with controls.

Residual diagnostics showed deviation from normality (Shapiro–Wilk $W = 0.9897$, $p = 0.002$), but given the large sample size, model estimates were considered robust.

Table 1
Demographic, biochemical and clinical characteristics of MG patients.

Demographic	
N	137
Sex, M (%)	49%
Age, years	64 (22–90)
Clinical	
Age at onset, years	52 (41–62)
Type, generalized:ocular	67%:33%
MGFA at onset	
I	29%
II	48%
III	16%
IV	6%
V	1%
Thymoma	18%
Thymic hyperplasia	12%
Thyreopathy	23%
Autoimmune disease	21%
Kidney disease	8%
Neuropathy	14%
Hypertension	38%
Cardiovascular disease	15%
Osteoporosis	26%
Eye disease	12%
Gastrointestinal disease	15%
Diabetes	12%
Haematological disease	8%
Cancer disease	7%
Psychiatric disorder	14%
Respiratory disease	11%
Neurological comorbidities	22%
Pyridostigmine	74%
Prednisone	73%
Biochemical	
anti-AChR title	9.51 (0.20 – >20)
anti-AChR pos	75%
anti-MuSK title	0.16 (0.00–1.49)
anti-MuSK pos	6%
anti-Lrp4 pos	0%
SN (seronegative)	19%

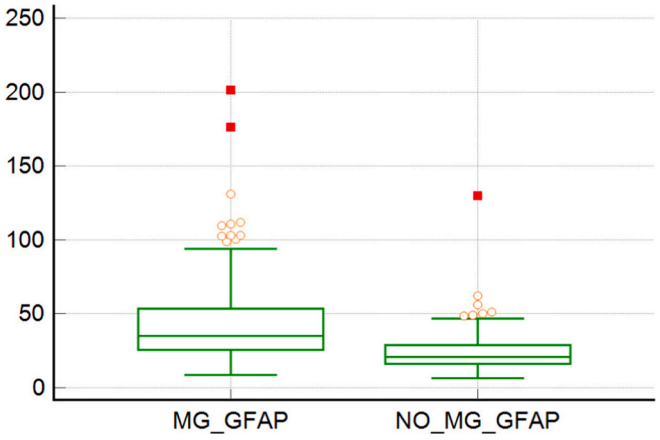

Fig. 1. Serum GFAP concentrations in patients with MG and controls.

Serum NfL concentrations were assessed in age- and sex-matched MG patients and controls (MG $n = 130$; controls $n = 132$). Median NfL levels were higher in MG patients (16.32 pg/mL [95% CI: 14.13–21.79]) compared with controls (13.07 pg/mL [95% CI: 11.93–15.79]). This difference was statistically significant (Mann–Whitney $U = 1397.0$, $Z = -2.37$, $p = 0.0177$), with a Hodges–Lehmann median difference of −3.51 pg/mL (95% CI: −7.00 to −0.54). However, substantial overlap between groups was observed, as reflected by similar interquartile ranges (MG: 11.40–28.36 pg/mL; controls: 10.71–19.63 pg/mL),

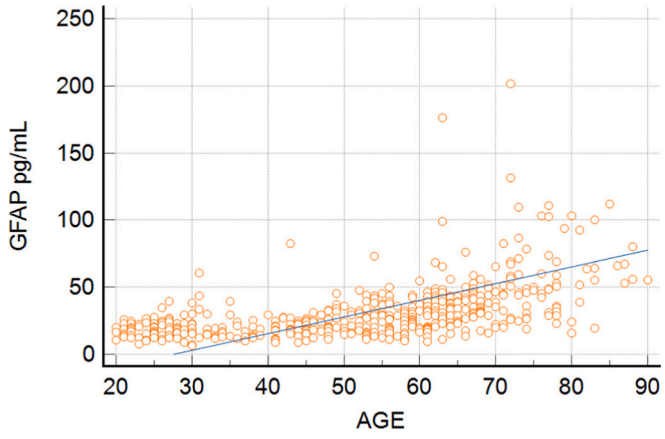

Fig. 2. Correlation between age and serum GFAPconcentrations in the overall study population.

indicating considerable variability and a limited discriminatory capacity of NfL alone.

Correlation analysis demonstrated a moderate positive association between serum GFAP and NfL concentrations in both MG patients and healthy controls. In MG patients, a moderate positive correlation was observed between serum GFAP and NfL concentrations, indicating a partial association between astroglial activation and neuroaxonal injury. A similar correlation was also observed in healthy controls ($r = 0.4804$, 95% CI 0.2388–0.6660; $p = 0.0003$). The presence of a similar correlation pattern in both MG patients and controls argues against a disease-specific interaction and instead supports a common physiological linkage between astroglial and neuroaxonal biomarkers.

Finally, ROC curve analysis showed that GFAP had moderate diagnostic performance in distinguishing MG patients from controls (AUC = 0.704, 95% CI 0.660–0.744; $p < 0.0001$), with an optimal cut-off value identified by the Youden index of 29.1 pg/mL, corresponding to a sensitivity of 69.3% and specificity of 68.1%. Conversly, NfL demonstrated lower discriminative ability and the combined use of GFAP and NfL did not significantly improve diagnostic accuracy compared with GFAP alone.

Overall, these findings indicate that neither GFAP nor NfL provides adequate diagnostic performance for distinguishing MG patients from controls in a matched population, and that NfL does not significantly outperform GFAP.

4. Discussion

In this cross-sectional study including 475 individuals, we evaluated serum GFAP and NfL concentrations in patients with MG compared with controls and explored demographic, clinical, and serological determinants of this biomarker.

We found that serum GFAP concentrations are elevated in patients with MG compared with controls (44.6 pg/mL vs 29.64 pg/mL), representing one of the first investigations of circulating GFAP levels in this neuromuscular junction disorder. Although serum GFAP concentrations were higher in MG patients compared with controls in unadjusted analyses, multivariable regression demonstrated that this difference was not independently attributable to MG status.

However, multivariate regression analysis revealed that this elevation is not independently attributable to MG status itself but rather reflects the strong confounding effects of age and sex. Age emerged as the dominant determinant of circulating GFAP ($\beta = 0.00827$, p 0.0001), with an approximate 0.83% increase per year, while female sex was associated with approximately 12% higher levels ($\beta = 0.114$, p 0.0001). A modest but statistically significant age × group interaction ($\beta = 0.00264$, $p = 0.044$) suggests that GFAP levels increase slightly more steeply with age in MG patients than in controls, though the clinical significance of this finding remains uncertain. The MG cohort exhibited a higher burden of comorbidities, including cardiovascular, metabolic, and neurological conditions, which are known to influence circulating GFAP levels. Therefore, the observed elevation of GFAP in MG patients is likely driven by demographic factors and comorbidities rather than disease-specific mechanisms.

GFAP is a major cytoskeletal intermediate filament protein expressed predominantly by astrocytes in the CNS [11,12,28–30].

In MG, circulating GFAP is independent of the autoimmune activity associated with MG, as supported by the lack of correlation between GFAP levels and anti-AChR or anti-MuSK antibody titers. On the other hand, the strong positive correlation between age and GFAP observed in our study is consistent with multiple recent investigations establishing age-stratified reference intervals for serum and plasma GFAP [27,31–34]. Several studies have documented a nonlinear increase in GFAP concentrations with advancing age, with particularly pronounced elevations after age 60 years [27,33]. For instance, Agnello et al. reported reference intervals of 10.4–92.0 ng/L in a general population, with significantly higher levels in individuals aged >60 years compared with younger age groups [27]. Similarly, Tybirk et al. established age-partitioned reference intervals showing progressive increases from 25 to 136 ng/L (ages 20–39) to 34–242 ng/L (ages 40–64) to 5–438 ng/L (ages 65–90) [33]. It should be noted that previously reported GFAP reference intervals are based on healthy populations and may not be directly comparable to cohorts with underlying disease and comorbidities.

The sex difference observed in our study, with females demonstrating approximately 12% higher GFAP levels, also aligns with emerging evidence. Agnello et al. reported higher GFAP levels in females across all age groups, with particularly pronounced differences in the 50–60 year age range [27]. Arslan et al. similarly documented sex-related differences after age 50, with females showing higher absolute GFAP levels than males [32]. The biological mechanisms underlying these sex differences remain incompletely understood but may relate to hormonal influences on astrocyte biology or sex-specific differences in blood-brain barrier permeability [34,35].

The finding that MG status does not independently influence GFAP levels has important implications for the potential clinical utility of this biomarker in MG. Unlike in Alzheimer's disease, where plasma GFAP demonstrates excellent diagnostic accuracy (AUC 0.97 in early-onset cases) and reflects underlying amyloid-β pathology, or in autoimmune GFAP astrocytopathy, where CSF GFAP antibodies are pathognomonic, circulating GFAP appears to lack disease specificity in MG [36–38].

While the age × group interaction reached statistical significance, its effect size was small and contributed only marginally to the overall variance, suggesting limited clinical relevance.

Alongside GFAP, we examined circulating NfL as a marker of neuroaxonal injury. In recent years, growing interest has focused on the potential role of NfL in MG, although the available evidence remains somewhat inconsistent. For instance, Stascheit et al. [39] reported an association between serum NfL levels and treatment response under intensified therapy, suggesting a role in capturing dynamic changes in disease activity rather than baseline disease characteristics. Similarly, Liu et al. [40] observed higher NfL levels in symptomatic compared with asymptomatic patients, pointing to a potential link with clinical status. However, these findings are not consistent, as earlier work by Stascheit et al. [41] highlighted considerable variability in NfL levels across MG subgroups, with limited ability to reliably distinguish between different clinical phenotypes.

In line with literature, our results showed only a modest increase in NfL levels in MG patients, accompanied by limited diagnostic performance. This suggests that, although NfL may reflect subtle neuroaxonal involvement, it does not represent a robust or disease-specific biomarker in MG.

Interestingly, a moderate positive correlation between GFAP and NfL

was observed in both MG patients and healthy controls. The presence of this association across groups suggests that it is not disease-specific, but rather reflects a shared underlying biological process, potentially related to physiological aging or baseline neurobiological turnover. The moderate strength of the correlation further supports the view that GFAP and NfL capture complementary, rather than overlapping, aspects of neurobiology.

ROC curve analysis further highlighted the limited diagnostic utility of these biomarkers. While GFAP initially appeared to show moderate discriminative ability in the overall cohort, its performance markedly decreased after age and sex matching, indicating that its apparent diagnostic value is largely driven by demographic differences. NfL showed only modest discriminative ability, and no significant difference was observed between the two markers. Overall, these findings indicate that neither GFAP nor NfL provides sufficient accuracy for distinguishing MG patients from controls when major confounding factors are considered.

Taken together, our results support the interpretation that circulating GFAP and NfL primarily reflect non-specific biological processes rather than disease-specific mechanisms in MG.

Several limitations of this study warrant consideration. First, the control group was younger than the MG cohort (median age 49 vs 64 years), which may have contributed to the observed group differences in unadjusted analyses. Although multivariate regression was employed to adjust for this imbalance, residual confounding cannot be entirely excluded. Second, the cross-sectional design precludes assessment of longitudinal biomarkers trajectories or potential associations with disease progression, treatment response, or clinical outcomes.

The lack of MG-specific GFAP elevation contrasts with findings in other neurological conditions. In neurodegenerative diseases such as Alzheimer's disease, frontotemporal dementia, and dementia with Lewy bodies, serum GFAP levels are substantially elevated and correlate with disease severity, cognitive decline, and neuropathological burden [42]. In multiple sclerosis and neuromyelitis optica spectrum disorder, GFAP levels reflect disease activity and predict disability progression [43]. In acute brain injuries such as traumatic brain injury and stroke, GFAP elevations are dramatic and have established clinical utility for diagnosis and prognosis [44].

The absence of disease-specific GFAP elevation in MG is consistent with its pathophysiology as a disorder of the neuromuscular junction rather than a primary CNS disease Although emerging evidence suggests the presence of systemic immune activation in MG, indicating by elevated circulating pro-inflammatory cytokines that may potentially contribute to low-grade neuroinflammation or astrocytic stress, these effects appear insufficient to produce a consistent, MG-specific biomarker signature. Future research should explore whether GFAP levels correlate with specific MG phenotypes, such as MG crisis, bulbar involvement, or treatment-refractory disease, where CNS or brainstem involvement might be more prominent. Longitudinal studies examining GFAP trajectories during disease exacerbations, remissions, and treatment interventions would help clarify whether dynamic changes in GFAP have prognostic value even if baseline levels lack diagnostic specificity.

CRediT authorship contribution statement

Caterina Maria Gambino: Writing – original draft, Investigation, Conceptualization. **Luisa Agnello:** Writing – review & editing, Investigation. **Concetta Scazzone:** Writing – original draft, Investigation. **Martina Tamburello:** Formal analysis. **Anna Masucci:** Formal analysis. **Roberta Vassallo:** Formal analysis. **Vincenzo Di Stefano:** Data curation. **Filippo Brighina:** Data curation. **Marcello Ciaccio:** Writing – review & editing, Visualization, Validation, Supervision, Investigation, Conceptualization.

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Declaration of competing interest

There are no conflicts of interest.

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None.

Data availability

Data will be made available on request.

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