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Insights from CD71 presentation and serum lipid peroxidation in myasthenia gravis – A small cohort study

Sinem Tunçer Çağlayan [a,*], Birsen Elibol [b], Feride Severcan [c], Esra Basar Gursoy [d,1], Bedile Irem Tiftikcioglu [e], Zeynep Gungordu Dalar [f,2], Ceren Celik [g], Ayse Suna Dai [h], Sevinç Karaçam [i]

[a] Bilecik Şeyh Edebali University, Vocational School of Health Services, Department of Medical Services and Techniques, Bilecik, Turkey
[b] Istanbul Medeniyet University, Faculty of Medicine, Department of Medical Biology, Istanbul, Turkey
[c] Altınbaş University, Faculty of Medicine, Department of Biophysics, Istanbul, Turkey
[d] Bezmialem Vakıf University, Faculty of Medicine, Department of Neurology, Istanbul, Turkey
[e] Izmir Bakırçay University, Faculty of Medicine, Department of Neurology, Izmir, Turkey
[f] Altınbaş University, Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkey
[g] Altınbaş University, Institute of Graduate Studies, Biomedical Sciences Graduate Program, Istanbul, Turkey
[h] Istanbul University, Faculty of Science, Department of Biology, Istanbul, Turkey
[i] Bilecik Şeyh Edebali University, Department of Biotechnology, Bilecik, Turkey

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ABSTRACT

Myasthenia gravis (MG) is a multifaceted autoimmune disorder affecting the postsynaptic neuromuscular junction. In this study, we examined $CD4^+$ and $CD8^+$ T lymphocyte levels and ratios within peripheral blood mononuclear cells (PBMCs) in MG patients. Additionally, we assessed lymphocytes for the expression of CD71, which functions as a transferrin receptor mediating the uptake of iron into the cells. Building on recent discussions regarding CD20 depletion treatments in MG, we also scrutinized lymphocytes for CD20 expression. Comparative analyses were conducted among healthy controls, newly diagnosed MG patients, those undergoing pyridostigmine treatment alone, and MG patients receiving combination therapies. In the patients, the ratio of $CD3^+CD4^+$ T lymphocytes to $CD3^+$ T lymphocytes was found to be decreased compared to the healthy controls, while the ratio of $CD3^+CD8^+$ cells to $CD3^+CD4^+$ cells increased. An increase in the percentage of CD71-expressing lymphocytes was observed in MG patients compared to the healthy control group, while $CD20^+$ lymphocytes exhibited no statistical changes. Moreover, heightened serum lipid peroxidation levels were found in MG patients. These results suggest a possible relationship between iron metabolism, levels of CD71-expressing cells, and lipid peroxidation in MG. Conversely, pyridostigmine treatment reduced the levels of CD71-expressing cells and lipid peroxidation, suggesting potential immunomodulatory and antioxidant impacts of pyridostigmine in MG, either directly or indirectly.

1. Introduction

Myasthenia gravis (MG), derived from Greek roots meaning muscle (myos) weakness (asthenos), and severe (gravis) in Latin, represents an autoimmune condition affecting the postsynaptic neuromuscular junction (NMJ). It serves as a prototypical instance of an autoimmune disease primarily mediated by antibodies. In this context, autoantibodies of the immunoglobulin G (IgG) class specifically target antigens present on the cell surface or within the extracellular matrix. These processes result in compromised NMJ transmission, leading to variable muscle weakness and an elevated susceptibility to fatigue [1]. In a significant number of patients, weakness typically begins in ocular muscles, resulting in symptoms like ptosis (eyelid drooping) or diplopia (double vision). Within the first two years, the majority of patients progress to

* Corresponding author at: Bilecik Şeyh Edebali University, Vocational School of Health Services, Department of Medical Services and Techniques, Pelitözü Mah. Fatih Sultan Mehmet Bulvarı No:27, 11100 Bilecik, Turkey.
E-mail address: sinem.tuncer@bilecik.edu.tr (S. Tunçer Çağlayan).
[1] Current address: University of Health Sciences, Basaksehir Cam and Sakura City Hospital, Department of Neurology, Istanbul, Turkey.
[2] Current address: Biruni University, Faculty of Medicine, Department of Medical Microbiology, Istanbul, Turkey.

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generalized weakness that involves bulbar and limb muscles [1,2].

MG is categorized as a rare disease with the potential to appear at any age, with the incidence higher in women than men, with a ratio of three to two [3]. In MG, an antibody-mediated, immunologic attack directed at proteins in the postsynaptic membrane of the NMJ (acetylcholine receptors or receptor-associated proteins) inhibits the excitatory effects of the neurotransmitter acetylcholine (ACh) on nicotinic receptors [4]. The prevalent antibodies in individuals with MG are primarily targeted against the acetylcholine receptor (AChR), defining the classical seropositive MG phenotype. In contrast, antibodies for muscle-specific tyrosine kinase (MuSK) are identified in around 5 % of patients with seronegative AChR MG. The MuSK protein plays a role in both the reassembly of AChR and the maintenance of the NMJ. In MG patients where neither AChR nor MuSK antibodies are present, these patients are classified as having double-seronegative MG (dSNMG) [1,5].

The exact cause of the autoimmune response in MG is still unknown; however, abnormalities in the thymus gland, such as hyperplasia and neoplasia, can play a role, especially in patients with anti-AChR antibodies. Additionally, genetic predisposition has been suggested to influence the development of the disorder [6]. MG is described as a T cell-dependent, B cell-mediated autoimmune disease [7], and specific T cell subsets are reported to be required for long-term antibody responses, with cytokines secreted mainly from $CD4^+$ T cells regulating B cell antibody production [8]. On the other hand, in experimental autoimmune MG (EAMG), Zhang et al. showed that interferon-gamma (IFN-γ) and interleukin-4 (IL-4) expressing cells were reduced after depletion of $CD8^+$ T cells; moreover, $CD8^+$ T cell depletion suppressed the incidence and severity of EAMG, accompanied by lowered levels of anti-AChR IgG antibody concentrations and decreased numbers of cells secreting anti-AChR antibodies [9]. The same group also suggested that since both $CD4^+$ T and $CD8^+$ T cells can express the effector factors IFN-γ and IL-4, the development of the disease may involve both helper and cytotoxic T cells [10].

In this study, we analyzed the level and ratio of $CD4^+$ and $CD8^+$ T cells in peripheral blood mononuclear cells (PBMCs) of MG patients. Additionally, the lymphocytes were analyzed for the transferrin receptor CD71 in which the expression in mononuclear cells is directly related to activation, growth control, and cell proliferation [8]. Based on recent data discussing the effectiveness of CD20 depletion treatment strategies in MG [9], we evaluated lymphocytes for CD20 expression. Moreover, for the first time in the literature, we assessed the levels of serum lipid peroxidation in the patient groups compared to the healthy control group to determine the oxidative stress and antioxidant status of the MG patients. The patient data were also evaluated in relation to pyridostigmine (PYR) treatment. As a reversible cholinesterase inhibitor used in the symptomatic treatment of MG, PYR prevents ACh hydrolysis, leading to an accumulation of ACh at cholinergic synapses, enhancing neurotransmitter release at nerve endings and increasing motor tone [11].

2. Materials and methods

2.1. Study groups

The absence of detectable autoantibodies in some MG patients frequently results in a delayed diagnosis. Neurophysiological methods like repetitive nerve stimulation (RNS) and single-fiber electromyography (SFEMG) prove essential in establishing an accurate diagnosis of MG, including seronegative MG [5,12]. Pyridostigmine (PYR), commonly employed for symptomatic treatment in MG, but also serves a diagnostic purpose. The response to PYR has been reported as a valuable diagnostic tool, particularly for patients with negative antibodies [13–17]. In addition to PYR, individuals with MG require long-term immunosuppressive therapies, including corticosteroids, azathioprine, and mycophenolate mofetil. They may also receive high-dose corticosteroids, intravenous immunoglobulins, or plasma exchange (plasmapheresis) during acute relapses and MG crises [18].

In the study, for all patients with a suspicion of MG on admission, a standardized diagnostic and follow-up protocol for MG was implemented. This protocol included investigation with RNS and SFEMG in addition to routine physical and detailed neurological examinations [19]. The patient assessment included a thorough examination of detailed disease characteristics, medical history, and family background. Information regarding the ongoing medical treatments was also documented. The patients were also evaluated for the presence of anti-AChR and anti-MuSK antibodies [19] either in the hospital as a part of clinical examination or in our research laboratory using Enzyme-Linked Immunosorbent Assay (ELISA; Cat. No: MBS700824 for anti-AChR and Cat. No: MBS705762 for anti-MuSK, MyBioSource, San Diego, USA) following the manufacturer's instructions. The newly diagnosed patients, who did not receive any treatment, were included in the "MG" group, and MG patients under the PYR (Mestinon®) treatment were included in the "MG PYR" group in the study. Daily PYR dose and the treatment duration varied among the patients (PRY 60–300 mg/d, min–max). Finally, the patients using prednisolone and/or azathioprine in addition to PYR, and patients who have received intravenous immunoglobulin therapy-IVIg) were included in the "MG combination therapy" group (MG Comb. Treatment). The "Control" group was recruited from the healthy population with no known autoimmune or neurological diseases and were not taking medications or substances that could affect neuromuscular nerve signals. When indicated, newly diagnosed (no treatment) MG patients (MG group) were categorized depending on the autoantibody status: D1 (disease 1) for the patients negative for both anti-AChR and anti-MuSK antibodies and D2 (disease 2) for those positive for at least one of the autoantibodies.

For MG, the global prevalence rates range from 150 to 200 cases per million [20]. The nature of a small subpopulation of this rare disease makes the research studies further challenging when the subsets of the patient groups are considered, including classification based on clinical presentation, antibody status, age of onset, and sex [7]. Using G*Power 3.1.9.4, for a very large effect size [21,22] equivalent to $d = 1.2$ [23], α error probability 0.05, power 0.8, and allocation ratio (control/MG) 2, the sample size was calculated as 7 for the disease group and 13 for the healthy control group. In the study, 30 control and 17 newly diagnosed MG patients, who do not receive therapy, were analyzed. The distribution of patient groups and healthy controls is included in **Table 1S** and Tables 1-3.

The experimental protocol of this study was approved by the Istanbul Bezmialem Vakif University (Istanbul, Turkey) Ethics Committee (Approval No: 15.10.2018–6032). Informed consent was obtained from each participant.

2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were separated by using standard density gradient centrifugation using Lymphocyte Separation Medium (Cat no: LSM-A, Capricorn Scientific, Germany) following the manufacturer's instructions. Briefly, a total of 4 mL of control or patient blood was mixed with 4 mL cell culture grade phosphate-buffered saline (PBS) and gently transferred to a 15 mL Falcon tube containing 6 mL of Lymphocyte Separation Medium and centrifuged at 800 x*g* for 20 min. at room temperature. Following centrifugation, the cloudy phase was carefully collected and transferred to a new 15 mL Falcon tube. PBS was added to complete the tube volume and the cells were washed twice at room temperature by centrifugation at 300 x*g* for 5 min. At the end of the centrifugation, the supernatant was removed and the pellet was suspended in PBS. The cells were stained with trypan blue and counted for viability analysis.

2.3. Surface staining and flow cytometry analysis

For antibody treatments, $5x10^5$ live cells in 100 μL of PBS were added

Table 1
"MG" group used in the study (F: female, M: male, IVIg: intravenous immunoglobulin therapy).

Patient	Age/Sex	MG type	Anti-AchR Ab	Anti-MuSK Ab	Pyridostigmine	Prednisolone	Azathioprine	Mycophenolate Mofetil	Other immunosuppressants	IVIg	Plasmapheresis
1	24/F	Ocular	(−)	(−)	No	No	No	No	No	No	No
2	46/M	Ocular	(−)	(−)	No	No	No	No	No	No	No
3	25/F	Ocular	(−)	(−)	No	No	No	No	No	No	No
4	39/M	Ocular	(−)	(−)	No	No	No	No	No	No	No
5	56/F	Ocular	(−)	(−)	No	No	No	No	No	No	No
6	18/M	Generalized	(−)	(−)	No	No	No	No	No	No	No
7	58/F	Generalized	(−)	(−)	No	No	No	No	No	No	No
8	49/F	Generalized	(−)	(−)	No	No	No	No	No	No	No
9	21/F	Generalized	(−)	(−)	No	No	No	No	No	No	No
10	35/M	Ocular	(−)	(−)	No	No	No	No	No	No	No
11	73/F	Generalized	(+)	(−)	No	No	No	No	No	No	No
12	28/F	Generalized	(+)	(−)	No	No	No	No	No	No	No
13	37/F	Generalized	(+)	(−)	No	No	No	No	No	No	No
14	53/F	Generalized	(+)	(−)	No	No	No	No	No	No	No
15	35/F	Generalized	(+)	(−)	No	No	No	No	No	No	No
16	31/F	Generalized	(−)	(+)	No	No	No	No	No	No	No
17	46/F	Ocular	(+)	(+)	No	No	No	No	No	No	No

Table 2
"MG PYR" group, consists of MG patients under pyridostigmine (PYR) treatment (F: female, M: male, IVIg: intravenous immunoglobulin therapy).

Patient	Age/Sex	MG type	Anti-AchR Ab	Anti-MuSK Ab	Pyridostigmine	Prednisolone	Azathioprine	Mycophenolate Mofetil	Other immunosuppressants	IVIg	Plasmapheresis
1	50/M	Ocular	(−)	(−)	Yes	No	No	No	No	No	No
2	56/F	Ocular	(−)	(−)	Yes	No	No	No	No	No	No
3	59/M	Ocular	(−)	(−)	Yes	No	No	No	No	No	No
4	59/M	Ocular	(−)	(−)	Yes	No	No	No	No	No	No
5	56/F	Ocular	(−)	(−)	Yes	No	No	No	No	No	No
6	41/F	Generalized	(−)	(−)	Yes	No	No	No	No	No	No
7	19/F	Ocular	(−)	(−)	Yes	No	No	No	No	No	No
8	81/F	Ocular	(−)	(+)	Yes	No	No	No	No	No	No
9	60/F	Generalized	(+)	(−)	Yes	No	No	No	No	No	No
10	29/M	Generalized	(+)	(−)	Yes	No	No	No	No	No	No

Table 3
MG patients under combination therapy, are categorized as the "MG Comb. Treatment" group (F: female, M: male, IVIg: intravenous immunoglobulin therapy).

Patient	Age/Sex	MG type	Anti-AchR Ab	Anti-MuSK Ab	Pyridostigmine	Prednisolone	Azathioprine	Mycophenolate Mofetil	Other immunosuppressants	IVIg	Plasmapheresis
1	49/M	Ocular	(−)	(−)	Yes	Yes	No	No	No	No	No
2	27/F	Ocular	(−)	(−)	Yes	Yes	No	No	No	No	No
3	26/F	Ocular	(−)	(−)	Yes	Yes	No	No	No	No	No
4	32/F	Generalized	(+)	(−)	Yes	Yes	No	No	No	Yes −3 years ago	No
5	73/F	Generalized	(+)	(−)	Yes	Yes	No	No	No	No	No
6	45/F	Generalized	(+)	(−)	Yes	Yes	No	No	No	No	No
7	50/M	Generalized	(+)	(−)	Yes	Yes	No	No	No	Yes −2 years ago	No
8	68/F	Generalized	(+)	(−)	Yes	Yes	No	No	No	No	No
9	69/M	Generalized	(+)	(−)	Yes	Yes	No	No	No	No	No
10	63/M	Ocular	(+)	(+)	Yes	Yes	No	No	No	No	No
11	63/M	Generalized	(−)	(−)	Yes	No	Yes	No	No	No	No
12	74/M	Generalized	(+)	(−)	Yes	No	Yes	No	No	No	No
13	63/M	Ocular	(+)	(−)	Yes	Yes	Yes	No	No	No	No
14	54/F	Generalized	(+)	(−)	Yes	Yes	Yes	No	No	No	No
15	69/M	Generalized	(+)	(−)	Yes	Yes	Yes	No	No	No	No
16	80/M	Generalized	(+)	(−)	Yes	Yes	Yes	No	No	Yes −2 years ago	No
17	61/M	Generalized	(+)	(−)	Yes	Yes	Yes	No	No	Yes	No
18	65/M	Generalized	(+)	(−)	Yes	Yes	Yes	No	No	No	No
19	33/M	Generalized	(+)	(−)	Yes	Yes	Yes	No	No	Yes −1 years ago	No
20	48/F	Generalized	(+)	(+)	Yes	Yes	Yes	No	No	Yes −6 years ago	No
21	74/M	Generalized	(−)	(−)	Yes	No	Yes	No	No	Yes	No
22	66/M	Generalized	(−)	(−)	Yes	No	Yes	No	No	Yes	No
23	42/F	Generalized	(+)	(−)	Yes	Yes	Yes	No	No	Yes	No
24	61/F	Generalized	(+)	(−)	Yes	No	No	No	No	Yes	No
25	60/F	Generalized	(+)	(−)	Yes	Yes	No	No	No	Yes	No
26	61/F	Generalized	(+)	(−)	Yes	No	No	No	No	Yes	No
27	30/M	Generalized	(+)	(−)	Yes	Yes	Yes	No	No	Yes	No

to each tube. The cells incubated 5 μL of the target antibodies or their respective isotypes. After 20 min. of incubation in the dark at room temperature, the samples were analyzed with BD Accuri C6 (BD Biosciences, USA). 10,000 events were gated for CD3, CD4 and CD8 analysis and 20,000 events were gated for CD20 and CD71 stainings. Lymphocytes were selected based on cell size and granularity and percentage distributions were determined based on the isotype gates. The antibodies and corresponding isotypes used in the study are listed in **Table 2S**. **Fig. 1S** presents a demonstration to illustrate the gating, isotype control, and target surface marker determination strategy employed in this study.

2.4. Thiobarbituric acid reactive assay

Blood samples were collected into red-top tubes containing clot activator but no anticoagulants, preservatives, or separator material. After clotting, serum was separated by centrifugation at 3000 rpm, for 10 min [24] and aliquated and stored at −80 °C. The thiobarbituric acid reactive substance (TBARS) assay was performed to determine the malondialdehyde (MDA) concentration as a measure of lipid peroxidation. A commercial calorimetric TBARS microplate assay kit (Biorbyt Ltd, Cambridge, UK) was used following the manufacturer's instructions. Briefly, serum samples were equilibrated to room temperature. After mixing the samples with assay buffer, they were centrifuged at 12,000 x*g* for 10 min at 4 °C. The supernatants were collected in new microcentrifuge tubes, and the dye reagent was added. Finally, the optical absorbances (OD) of each well were measured at 535 nm using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Boston, MA, USA). Two technical replicates were applied for each sample and the levels of MDA concentration were determined as μmol/L using a standard curve.

2.5. Statistical analysis

The results were presented as mean ± SD (standard deviation) or mean ± SEM (standard error of the mean) and pairwise comparisons of the groups have been conducted using unpaired *t*-test. Significance levels were presented as *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, **** $p \leq 0.0001$. GraphPad Prism 8.0 was used for the creation of graphs and statistical comparisons ($p \leq 0.05$).

3. Results and discussion

3.1. T cell subset levels change in myasthenia gravis patients

The percentage of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T lymphocytes and the percentage of lymphocytes expressing CD71, CD20, and both are given in Table 4. Although it did not reach statistical significance, based on the decreasing trend in the percentage of $CD3^+$ and $CD3^+CD4^+$ cells in the MG patient group, the ratio of the $CD4^+$ T lymphocytes (%) and $CD8^+$ T lymphocytes (%) to $CD3^+$ (%) cells was calculated and the results are shown in Fig. 1**A** and Fig. 1**B**, respectively. In Fig. 1**C**, the ratio of $CD3^+CD8^+$ cells (%) to $CD3^+CD4^+$ cells (%) is given. Accordingly, in MG patients, the ratio of $CD4^+$ T lymphocytes to $CD3^+$ T lymphocytes decreases significantly compared to the healthy control group, while the ratio of $CD3^+CD8^+$ cells to $CD3^+CD4^+$ cells increases. These effects appear independently from the autoantibody status (D1 and D2 groups) of the patients (**Fig. 2S**). On the other hand, no significant difference was observed in the MG patients under pyridostigmine (PYR) treatment (MG PYR group) and in MG patients receiving combined treatment (MG Comb. Treatment group) compared with the control group.

Previously, a decrease in the $CD3^+CD4^+$ population was observed in both AChR-positive and dSnMG patients [8]. Although most studies on MG pathogenesis have predominantly focused on $CD4^+$ T cells, further studies are needed to focus more deeply on examining the roles of $CD8^+$ T cells in the disease etiology or progression [25]. Li et al. recently claimed that $CD8^+$ T cells demonstrate an effector phenotype with prominent polyfunctional inflammatory cytokine function in MG patients and may potentially serve as a biomarker for monitoring response to steroid treatment [26]. Additionally, an earlier study reported a significant association with the MHC class I allele HLA B8, which affects susceptibility, immunoregulation, and clinical expression in MG [27]. In this context, considering MHC class I as the main restriction element for $CD8^+$ T cells [10], typing human leukocyte antigen (HLA) genes can be valuable for MG diagnosis and subgrouping. These investigations have the potential to suggest functional correlates and distinct predisposing mechanisms influenced by genetic factors (such as the implication of the HLA genes or the gene for protein tyrosine phosphatase nonreceptor type 22, PTPN22), in addition to environmental factors (such as viral infections) [28–31]. Besides being important for both clinical and basic research, this approach can be valuable for a better understanding of the disease and potentially leads to more targeted treatments [30].

In addition to the change in $CD4^+$ to $CD8^+$ T cell ratio, our results also suggest a potential restorative effect of PYR treatment, revealing a trend towards enhancing circulating $CD4^+$ T lymphocytes in MG for the first time. The restoration phenomenon was previously observed in Human Immunodeficiency Virus (HIV)-infected patients undergoing PYR treatment [32]. This effect of PYR may be attributed to the immunomodulatory action of ACh. Lymphocytes isolated from the thymus, lymph nodes, spleen, and peripheral blood express both muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively). Components of the cholinergic system, including ACh, choline acetyltransferase (ChAT), acetylcholinesterase, and both mAChRs and nAChRs, are present in T and B cells, suggesting cholinergic involvement in the regulation of immune function [33,34]. It has been demonstrated that nAChR signaling plays a role in regulating cytokine production, modulating antibody production, and contributing to immunomodulation by modifying T-cell differentiation [34,35]. Additionally, nicotinic stimulation of thymic epithelial cells by ACh, synthesized and released by thymocytes, was shown to protect thymocytes from apoptosis and promote their differentiation [33]. Ultimately, research on peripheral blood T cells and the Jurkat T cell leukemia line demonstrated that nAChR stimulation can directly alter lymphocyte function and also affect it through interactions with the surrounding microenvironment, partially explaining its wide-ranging impact on cellular survival [36]. Thus, it can be suggested that the increase in the half-life of ACh due to PYR may impact the composition of circulating T lymphocyte subsets.

3.2. The percentages of CD71-expressing lymphocytes are elevated in myasthenia gravis patients and pyridostigmine treatment can reverse this effect

Transferrin is an abundant plasma protein that binds iron and delivers it to cells through its receptor CD71, following binding and

Table 4
Surface marker expressions presented as mean ± SD.

Group	%$CD3^+$	%$CD3^+$ $CD4^+$	%$CD3^+CD8^+$	%$CD71^+$	%$CD20^+$	%$CD20^+CD71^+$
Control	67.04 ± 6.07	42.38 ± 6.38	20.57 ± 5.89	0.77 ± 1.05	9.11 ± 2.85	9.1 ± 2.84
MG	61.73 ± 16.65	35.46 ± 11.27	20.36 ± 7.49	5.87 ± 5.67	8.89 ± 4.33	8.67 ± 4.31
MG PYR	64.89 ± 12.85	40.51 ± 9.36	21.38 ± 6.36	0.73 ± 1.50	9.05 ± 4.31	7.62 ± 3.07
MG Comb. Treatment	62.14 ± 15.13	39.90 ± 15.05	19.00 ± 9.38	10.14 ± 10.67	9.40 ± 8.99	8.64 ± 7.09

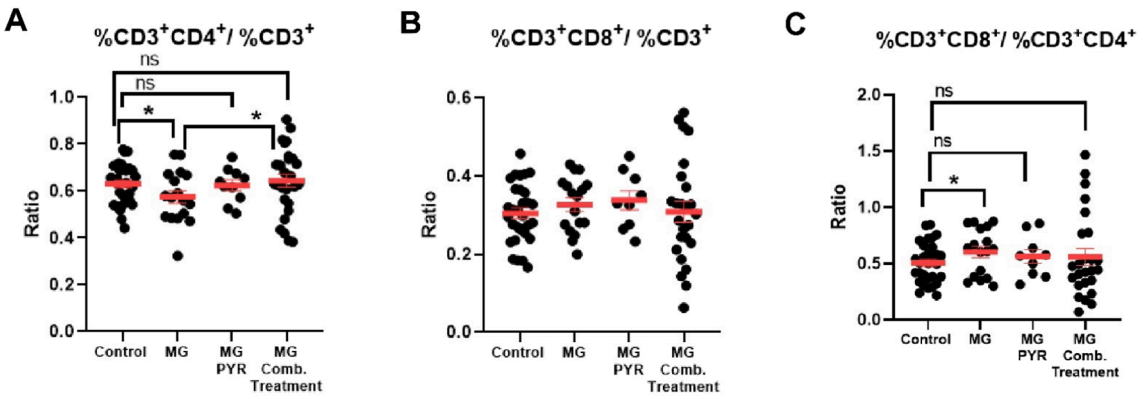

Fig. 1. The ratio of $CD4^+$ T lymphocytes decreases in MG patients but is reversed with disease therapy. The ratios of the frequency of circulating A. $CD3^+CD4^+$ lymphocytes and B. $CD3^+CD8^+$ to T lymphocytes, as well as C. the ratio of % $CD3^+CD8^+$ T cells to % $CD3^+CD4^+$ T cells are presented. The data are shown as mean ± SEM. *t*-test was employed for comparisons with the control group and among the patient groups as indicated (*p ≤ 0.05, ns = non-significant). Each data point represents an individual.

internalization. This transferrin/CD71 system is particularly crucial for erythroblasts and lymphocytes, including T and B cells. CD71 is either absent or expressed at low levels on non-proliferating cells but undergoes rapid upregulation upon cellular activation and growth [37]. As shown in Fig. 2, there is a significant increase in the percentage of lymphocytes expressing CD71 in MG patients compared to the healthy control group independent from the antibody status (**Fig. 3S**). However, PYR treatment reversed this effect. As given in Table 4, both the percentage of $CD20^+$ expressing cells and the quantity of $CD20^+CD71^+$ cells exhibited no statistically significant changes when compared to the control group, as well as between the different patient groups. Consequently, the CD71-expressing cells in the MG group may potentially indicate proliferating/activated T lymphocytes. This result contradicts an early report suggesting elevated numbers of $CD71^+$ B cells in MG [38], a finding that could not be substantiated by other studies [39].

In the context of MG, the involvement of B cells is more complex than initially anticipated, given the contribution of a heterogeneous population of B cells to the disease's etiology [40]. Within this group of cells, plasmablasts are short-lived, proliferate, and, to a small extent, contribute to autoantibody production. These cells eventually transform into long-lived plasma cells with a high capacity for antibody production [41]. Long-lived plasma cells produce a substantial amount of circulating immunoglobulins but do not express CD20 on their surfaces. This indicates also the need for careful evaluation of the effectiveness of strategies targeting CD20, such as monoclonal antibody therapy, in the treatment of MG [40].

3.3. Serum lipid peroxidation levels are elevated in myasthenia gravis patients and can be modulated by disease therapy

Finally, we assessed serum lipid peroxidation levels in the patient groups compared to the healthy control using the TBARS assay, which detects the level of MDA, a major lipid oxidation product and a crucial marker of oxidative stress and antioxidant status [42]. As depicted in Fig. 3 and **Fig. 4S**, lipid peroxidation was significantly elevated in MG patients compared to the control group. However, in patients treated with PYR, although still higher than the healthy control group, the level of peroxidation decreased significantly. In the group of patients undergoing combined immunosuppression therapy along with PYR (MG Comb. Treatment group), the serum lipid peroxidation level was notably higher than the control group but showed no statistical difference from the MG group. Additionally, though not reaching statistical significance, a trend toward higher MDA levels in the combined treatment group was

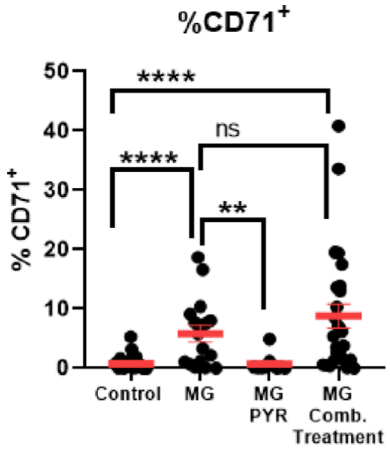

Fig. 2. The presence of circulating $CD71^+$ lymphocytes increases in MG patients, and this effect is reversed with pyridostigmine (PYR) treatment. % of the CD71 expressing lymphocytes are presented. Each data point corresponds to an individual. The results are presented as mean ± SEM and the *t*-test was used for comparisons with both the control group and among the patient groups as specified (**p ≤ 0.01, **** p ≤ 0.0001, ns = non-significant).

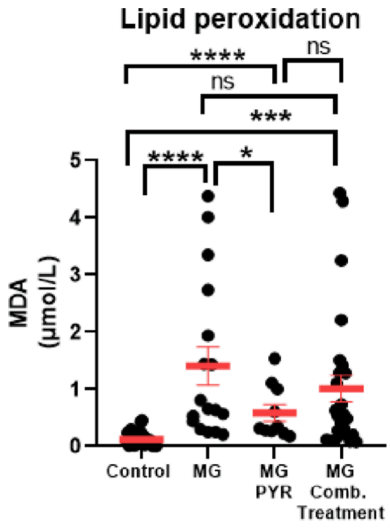

Fig. 3. Serum lipid peroxidation levels are elevated in MG patients and decrease with the therapy. Each data point represents an individual, and the results are displayed as mean ± SEM. The *t*-test was employed for comparisons with the control group and among the patient groups as specified (*p ≤ 0.05, ***p ≤ 0.001, **** p ≤ 0.0001, ns = non-significant).

apparent when compared with the MG PYR group.

3.4. CD71-expressing cells may be responsible for the elevated serum lipid peroxidation levels in myasthenia gravis

Many enzymes that play key roles in DNA replication, repair, and translation depend on iron, making it especially critical for proliferating cells. Transferrin binds to iron and transports it into cells by interacting with and being internalized through its receptor, CD71. This receptor is either absent or minimally expressed in non-proliferating lymphocytes but is upregulated during cellular activation and growth [36]. Huang reported that serum iron levels are decreased in MG patients [43] and Li et al. showed that compared with the healthy individuals, the non-anemic patients with MG had significantly lower levels of serum iron and transferrin saturation, and a significantly larger proportion of the patients with MG had serum iron and transferrin saturation levels below the normal range [44]. More recently, ferritin levels were reported to be positively correlated with disease severity, and total iron-binding capacity and transferrin levels correlated with outcomes in MG [45]. It was also claimed that iron metabolism parameters did not significantly change after approximately one year of immunotherapy in MG patients [44]. In systemic lupus erythematosus (SLE), another autoimmune disease, elevated CD71 levels were found in T lymphocytes and this causes the accumulation of iron in SLE T cells despite the predisposition of SLE patients to develop low serum iron [46], similar to MG. Here, enhanced CD71 expression in the lymphocytes of MG patients is reported, which may be responsible for the observed low serum iron levels in the patients. Interestingly, PYR treatment seems to reverse this effect.

CD71-mediated iron intake contributes to the increase of the intracellular Fe^{2+} pool (referred to as the labile iron pool). Iron participates in the Fenton reaction to generate reactive oxygen species (ROS). By initiating lipid peroxidation, ROS damages lipids and thus plays a role in promoting regulated cell death, including ferroptosis, an iron-dependent cell death. Lipid peroxides, a subset of ROS, act as signaling molecules that can modify cell membrane properties, lipid interactions, and protein functions [47,48]. The lipid peroxides formed during ferroptosis accumulate in the plasma membrane preceding cell rupture [49]. Oxidative stress, ferroptosis, and inflammation share a tight interconnection, potentially collaborating and mutually influencing each other [50]. Oxidative stress is evident in the progression of numerous diseases and contributes to the etiopathogenesis of certain conditions, including inflammatory and autoimmune diseases [51,52]. Highlighting the pathogenic impact of ROS, glycoxidation damage to blood serum proteins has been documented previously in MG patients positive for anti-AChR antibodies [53]. Besides, it seems that increased generation of ROS can cause inactivation of AChRs [54]. To our knowledge, this study represents the first analysis of serum lipid peroxidation levels in MG patients reported in the literature. Previously, Yang et al. demonstrated that serum levels of bilirubin, uric acid, albumin, and creatinine are reduced in patients with MG indicating an active oxidative process in MG patients who had low antioxidant status; although the relationship between antioxidant status and MG has not been fully discussed [55]. Similar results for bilirubin and uric acid were also reported by Fuhua et al. [56].

Intriguingly, the observed profile for serum lipid peroxidation is very similar to the CD71 expression results. In MG, iron-dependent cellular damage may be responsible for the increased levels of serum lipid peroxidation. The enhanced percentage of CD71-expressing cells (Fig. 2), regarded as one of the markers of ferroptosis [50,51], may contribute to the elevated serum lipid peroxidation levels (Fig. 3). On the other hand, the effect of PYR in decreasing the serum lipid peroxidation is well correlated with the decreased number of lymphocytes expressing CD71 with respect to the untreated MG group. In the ischemia/reperfusion (I/R)-induced oxidative stress model, ACh was shown to inhibit ROS production thus protecting cells against H/R-induced oxidative stress, as well as cell injury [57]. Therefore, it is needed to emphasize that the observed decrease in lipid peroxidation in PYR-treated MG patients compared to untreated patients may driven by the effect of PYR on the ACh level. In addition, a negative correlation between protein glycoxidation markers in MG with the dose of PYR was shown [53] as an indication of an antioxidant role for PYR. Thus, the role of oxidative stress in MG initiation and/or disease progression, together with the effect of PYR needed to be elucidated. Besides, the involvement of ROS, iron (considering CD71 expression), and ferroptosis in the pathogenesis of MG warrants further detailed investigation.

On the other hand, in patients under combination therapy, there was no significant difference in the percentages of lymphocytes expressing CD71 compared to the untreated MG group. It is important to note that the combination therapy group is very heterogeneous, as specified in Table 3. In addition to PYR treatment, these patients are subjected to immunosuppressive treatments such as the corticosteroid prednisolone and the purine metabolism antagonist azathioprine. Accordingly, Tillinger et al. demonstrated that in Crohn's disease, an inflammatory condition characterized by elevated levels of T cells expressing CD71 compared to healthy controls, Deltacortril® (prednisolone) treatment did not lower the percentage of $CD71^+$ T lymphocytes [58].

4. Conclusion

The standard therapies, including cholinesterase inhibitors, corticosteroids, immunosuppressive drugs, immunoglobulin, plasma exchange, and thymectomy, are effective for many MG patients, even though the pathogenic processes of MG are still not fully understood. However, challenges remain in treating MG. Some refractory patients do not respond to these traditional therapies, while others discontinue use due to adverse effects [59].

This study supports the concept of immune dysregulation in MG and indicates a possible relationship between iron metabolism and the levels of CD71-expressing cells in MG within the context of iron homeostasis. The observed changes in CD71 counts and lipid peroxidation levels highlight potential avenues for future research into the interplay of ROS, iron metabolism, and ferroptosis in MG initiation and progression, which could aid in the development of tailored therapeutic strategies. In this context, examining the therapeutic effects of antioxidants and ferroptosis inhibitors may provide crucial insights into the pathogenesis of the condition. Furthermore, in MG, understanding the mechanism of action of PYR requires a thorough investigation within the framework of immune-neurohumoral interactions, focusing on its potential immunomodulatory and direct or indirect antioxidant effects (Fig. 4).

Despite its original contributions to the knowledge, it is imperative to acknowledge certain limitations in this study, including the relatively small sample size and the absence of specific cell sub-population identification through the use of appropriate markers. Future investigations, encompassing a larger cohort and providing a more detailed profiling of immune cell subsets, are warranted for a comprehensive and in-depth analysis.

Lastly, several limitations should be emphasized regarding MG cohort studies. As previously pointed out, MG affects more women than men, with a ratio of three to two [3,60] and sex has been reported to influence clinical outcomes of the disease [61]. Additionally, while the disease can manifest at any age, current evidence suggests MG is more prevalent among young women and elderly men [60]. Furthermore, early-onset MG has also been reported to predominantly affect women, whereas late-onset MG tends to affect men [3]. Therefore, considering variations among patients (including differences in sex, age, disease pathophysiology, clinical features, as well as changes in treatment strategies from both patient and clinician perspectives) is crucial when designing cohort studies. On the other hand, it should also be noted that MG is a rare disorder, which makes it challenging to gather a sufficient number of patients to account for these differences and create balanced cohorts.

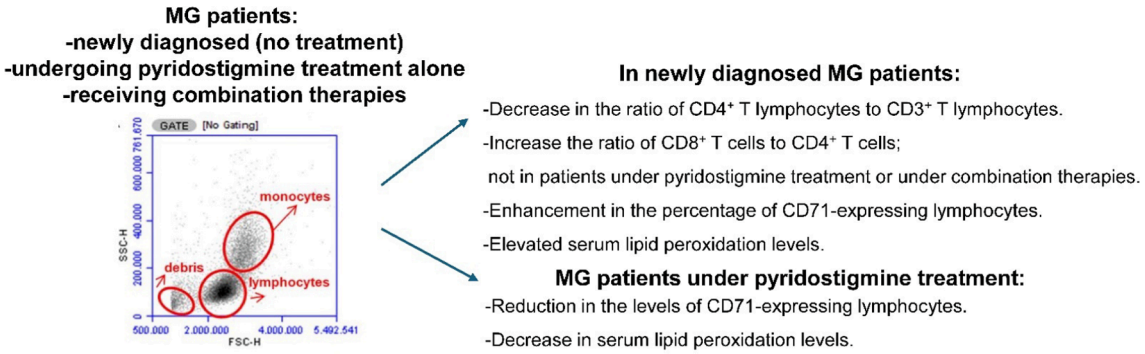

Fig. 4. Main results of the study are summarized. Immunological changes and changes in serum lipid peroxidation levels were observed in MG patients. Treatment status, including pyridostigmine (PYR) treatment, impacts the disease outcome.

Author contributions

STÇ, EBG, BIT, and FS conceptualized the study. EBG and BIT diagnosed the MG patients and collected clinical samples. BE supervised and conducted the flow cytometry experiments with ZGD, CC, and ASD, and carried out lipid peroxidation experiments. SK performed ELISA experiments with STÇ to determine antibody status. STÇ collected and analyzed the data, interpreted the results, and wrote the manuscript. FS organized and oversaw the study. All authors reviewed the results and approved the final version of the manuscript.

CRediT authorship contribution statement

Sinem Tunçer Çağlayan: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Birsen Elibol:** Supervision, Investigation, Data curation. **Feride Severcan:** Supervision, Funding acquisition, Conceptualization. **Esra Basar Gursoy:** Investigation, Conceptualization. **Bedile Irem Tiftikcioglu:** Investigation, Funding acquisition, Conceptualization. **Zeynep Gungordu Dalar:** Data curation. **Ceren Celik:** Data curation. **Ayse Suna Dai:** Data curation. **Sevinç Karaçam:** Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2024.112787.

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