Title Page

Title: Iron Metabolism in Non-anemic Myasthenia Gravis Patients: A Cohort Study

Author Names: Ke Li[1], Li'an Hou[2], Ying Tan[1], Yangyu Huang[1], Jiayu Shi[1], Jianhua Han[2], Jingwen Yan[1], Yuzhou Guan[1], Liying Cui[1]

Author Affiliations: [1] Department of Neurology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China 100730

[2] Department of Clinical Laboratory, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China 100730

ORCID: Ke Li, 0000-0002-7707-3158; Yuzhou Guan, 0000-0003-3822-4440

Corresponding Author Info: Yuzhou Guan, Department of Neurology, Peking Union Medical College Hospital, #1 Shuaifuyuan, Dongcheng District, Beijing, China. Telephone Number: +86-13910081750; E-mail: guanyz001@163.com.

Key Words: myasthenia gravis, iron metabolism disorders, ferritins, transferrin, survival analysis

Acknowledgement: None.

Iron Metabolism in Non-anemic Myasthenia Gravis Patients: A Cohort Study

Ke Li[1], Li'an Hou[2], Ying Tan[1], Yangyu Huang[1], Jiayu Shi[1], Jianhua Han[2], Jingwen Yan[1], Yuzhou Guan[1], Liying Cui[1]

[1] Department of Neurology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China 100730

[2] Department of Clinical Laboratory, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China 100730

Correspondence to Yuzhou Guan, Email: guanyz001@163.com

Abstract

Background: The association of iron metabolism parameters with disease severity and outcome in myasthenia gravis (MG) patients has not been reported. This study was conducted to determined clinical factors including iron metabolism parameters correlated with disease severity and future outcome in non-anemic immunotherapy-naïve MG patients first receiving immunotherapy.

Material and methods: One hundred and ten patients were included at baseline to explore predictor variables associated with disease severity represented by variables derived from MG activities of daily living (MG-ADL) score using multivariate logistic regression, after which 103 and 98 patients were included respectively in multivariate survival analyses at 6-month and 12-month follow-up to identify predictors for minimal manifestation status (MMS) after starting immunotherapy.

Results: Higher ferritin level was independently associated with higher risk of severe generalized disease in non-anemic immunotherapy-naïve MG patients. Total iron binding capacity < 250 μg/dL and the interval between onset and immunotherapy < 1 year were independent predictors for MMS at 6-month and 12-month follow-up after initiating immunotherapy. Transferrin < 2.00 g/L was an independent predictor for MMS at 12-month follow-up.

Conclusion: Iron metabolism parameters might be promising biomarkers for evaluating disease severity and guiding therapeutic decision in MG patients.

Key Words

myasthenia gravis, iron metabolism disorders, ferritins, transferrin, survival analysis

1 Introduction

Myasthenia gravis (MG) is an autoimmune disease caused by dysfunction of postsynaptic membrane at neuromuscular junction, featuring fluctuating weakness in skeletal muscles. The estimated prevalence rate of MG is 64.0-94.3 cases per million (Carr et al. , 2010). Common pathogenic autoantibodies are anti-acetylcholine receptor antibody (AChR-Ab) and anti-muscle-specific tyrosine kinase antibody (MuSK-Ab).

Iron is a microelement indispensable for physiological and pathophysiological processes of living organisms, involving in autoimmunity (Cronin et al. , 2019), tumorigenesis (Torti and Torti, 2013), and infection (Nairz and Weiss, 2020). In human nervous system, iron participates in myelination, neurotransmitter synthesis, oxidative phosphorylation and other crucial processes (Sfagos et al. , 2005). Recent studies have shown that iron metabolism disorders might be evident in neuroimmune diseases including multiple sclerosis (Abo-Krysha and Rashed, 2008, Sfagos, Makis, 2005, van Rensburg et al. , 2006), polymyositis/dermatomyositis (Gono et al. , 2010, Kawasumi et al. , 2014, Kobayashi et al. , 2017), and so on. However, to our knowledge, no published study has analyzed the association of iron metabolism parameters with disease severity and clinical outcome in MG patients.

The primary objectives of the research were to identify clinical factors associated with disease severity and minimal manifestation status (MMS) induction in non-anemic immunotherapy-naïve MG patients first receiving immunotherapy, with iron metabolism parameters specifically focused.

2 Material and methods

Our research was a prospective cohort study. In the first part of the study, clinical characteristics of eligible MG patients were analyzed to identify factors independently associated with disease severity. In the second part, patients were followed-up over specific periods of time after starting immunotherapy to explore factors predicting MMS. Reporting of the research adhered to the statement of Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) (von Elm et al. , 2007).

2.1 Subjects and outcomes

Our research recruited 110 MG patients from Peking Union Medical College Hospital (PUMCH), Beijing, China, from October 2015 to February 2022. Inclusion criteria were: 1) confirmed diagnosis of MG (typical fatigable muscle weakness with unequivocally positive pharmacological testing, electromyography studies, or MG-specific antibody assays) (Evoli et al. , 2019); 2) hemoglobin ≥ 120 g/L for male or ≥ 110 g/L for female, and not on iron supplementary therapy; 3) MMS not reached and immunotherapy required to induce MMS; 4) no histories of receiving immunotherapy, which refers to glucocorticoids and/or immunosuppressants. Exclusion criteria were: 1) comorbidity with clinically diagnosed malignant tumor; 2) comorbidity with severe cardiac, hepatic, or renal insufficiency; 3) negativity for AChR-Ab and MuSK-Ab with an onset age <13 years; 4) incomplete clinical data. Patients who refused immunotherapy were included in the first part of the study but excluded from subsequent follow-up. Immunotherapy was initiated at inclusion for eligible participants.

At baseline, we collected clinical characteristics and iron metabolism parameters of included patients, and performed cross-sectional analysis. We then followed up the patients who consented to receive immunotherapy for 6 and 12 months after initiating immunotherapy. Endpoint events (MMS) and follow-up time were included in the survival analyses. Patients usually came to MG clinic for follow-up every 1-3 months. For patients lost during follow-up, the last status was included in survival analyses, while those who merely had first visit were excluded. Follow-up of all patients ended on 31 May, 2022. Patients who did not reach

MMS on this date with insufficient follow-up time (6 months or 12 months) were excluded from corresponding survival analyses. Sensitivity analysis were performed to test the consistence of our results. Processes of the research were summarized in Fig. 1. Sample size calculation was not conducted beforehand since few previous data on the role of iron metabolism in MG were present.

Iron metabolism parameters involved were serum iron (SI) (reference range 65-175 μg/dL), transferrin (Tf) (reference range 2.00-3.60 g/L), total iron binding capacity (TIBC) (reference range 250-450 μg/dL), transferrin saturation (TS) (reference range 25.0-50.0%), and ferritin (24-336 ng/mL). Reference ranges were determined by the Department of Clinical Laboratory of PUMCH.

Exposure to a drug was defined as taking it for more than 3 weeks with a dose of 20-60 mg per day or equivalent for prednisone, or with blood concentration ≥ 4.8 ng/ml for tacrolimus (TAC). No patients in our cohort were exposed to azathioprine or mycophenolate mofetil. Intravenous methylprednisolone pulse (IVMP) therapy was defined as intravenous administration of methylprednisolone at a dose of 500-1000 mg per day for 3-5 days, and intravenous immunoglobulin (IVIg) was defined as intravenous administration of immunoglobulin at a dose of 0.4 g per kilogram body weight per day for 5 days.

As inflammation in MG might distort interpretation iron deficiency (ID), we defined ID as ferritin < 100 ng/ml, or TS < 20% if ferritin was 100-299 ng/ml (McDonagh et al. , 2021). Severe generalized disease (SGD) and estimated patient acceptable symptom status (ePASS) were derived from MG activities of daily living (MG-ADL) scores to quantify disease severity. SGD was defined as MG-ADL ≥ 6 with at least 1 point in non-ocular items (Petersson et al. , 2021), and negative ePASS was defined as MG-ADL ≥ 3, while positive ePASS meant MG-ADL < 3 (Mendoza et al. , 2020). MMS indicated absence of functional limitations from MG except for some weakness on examination (Jaretzki et al. , 2000).

2.2 Statistical Analysis

Numerical variables were displayed as mean ± standard deviation should they obey normal distribution, otherwise as median [interquartile range (IQR)]. When comparing two or more independent samples, parametric tests including Student's *t* test and one-way analysis of variance were preferred when appropriate, otherwise non-parametric tests including Mann-Whitney U test and Kruskal-Wallis test were used. For comparison of proportions, chi-square test was adopted.

To determined factors independently associated with SGD and negative ePASS, univariate and multivariate binary logistic regression models were used. Variables with a p value < 0.10 in the univariate analysis were included in the multivariate binary logistic regression after eliminating multicollinearity. When analyzing independent predictors for MMS induction, univariate survival analysis and Cox proportional hazards model were adopted. Predictors were dichotomized in univariate survival analysis, and those with a p value < 0.10 were included in the Cox proportional hazards model, with multicollinearity eliminated. Sensitivity analyses were conducted finally.

Significance level (α) was set at 0.05 (two-tailed). Odds ratio (OR) or hazard ratio (HR) with 95% confidence interval (CI)

were displayed when appropriate. Data analysis was facilitated by SPSS 26 (IBM, NY, USA).

2.3 Ethics statement

The research was performed according to the Declaration of Helsinki and approved by Ethics Committee of Clinical Research of PUMCH, Beijing, China. Written informed consents were obtained from all participants, their legal guardians, or next of kin.

3 Results

3.1 Baseline characteristics

A total of 116 immunotherapy-naïve MG patients were assessed for eligibility (Fig. 1). Three patients with anemia, 1 comorbid with cancer, 1 negative for AChR-Ab and MuSK-Ab with onset age < 13 years, and 1 with incomplete data were excluded. Four patients refused immunotherapy, and 3 did not come back for follow-up after initiating immunotherapy. Ultimately, 110 patients were included in the cross-sectional analysis at baseline and 103 remained in the survival analysis. Five non-MMS patients whose follow-up time fell between 6 to 12 months when follow-up ended on 31 May, 2022 were included in the 6-month survival analysis but excluded from the 12-month one.

The 110 patients consisted of 54 females, 26 of whom were premenopausal. The median onset age of MG was 51.0 years (IQR 31.0-61.3 years). The median interval between onset and immunotherapy was 12.1 months (IQR 4.3-37.6 months). Eighty-seven patients were positive for AChR-Ab, 7 positive for MuSK-Ab, and the others negative for both. As for Myasthenia Gravis Foundation of America (MGFA) clinical classification at nadir, the numbers of patients categorized as MGFA I, II, III, and above were respectively 36, 27, 37, and 10. Nineteen patients had histories of thymoma and 7 were comorbid with other autoimmune diseases, with 22 thymectomized before inclusion.

Hemoglobin and iron metabolism parameters were as follows: median hemoglobin 145 g/L (IQR 132-156 g/L), SI 95 ± 34 μg/dL, Tf 2.35 ± 0.42 g/L, TIBC 321 ± 62 μg/dL, median TS 29.3% (IQR 23.0-36.7%), median ferritin 94 ng/mL (IQR 42-170 ng/mL). Sixty-two patients reached our criteria for iron deficiency. Levels of hemoglobin and iron metabolism parameters were then compared across specific subgroups (Table 1). As for male, postmenopausal female and premenopausal female participants, their hemoglobin level, ferritin level, and ID rate were significantly different, with the latter having lower ferritin level and higher ID rate. Ferritin level were significantly different across subgroups of MGFA clinical classification. Specifically, ferritin level was significantly lower in premenopausal female and MGFA I patients than other subgroups (Fig. 2). Patients with SGD had significantly lower Tf, lower TIBC, higher ferritin, and lower rate of ID compared with those without SGD (Fig. 2). No significant difference was found between patients with negative ePASS and positive ePASS in hemoglobin or iron metabolism parameters involved.

Table 1 Iron metabolism parameters in MG patients.

	Hb (g/L)	SI (μg/dl)	Tf (g/L)	TS (%)	TIBC (μg/dl)	Ferritin (ng/ml)	ID (%)

Gender and	Male	153 ± 12	100 ± 28	2.29 ± 0.41	30.3 (24.0-37.2)	313 ± 58	123 (80-217)	37.5
menstrua	PostM F	136 ± 11	89 ± 34	2.37 ± 0.46	27.4 ± 10.8	320 ± 71	97 (41-155)	57.1
-tion	PreM F	134 ± 9	90 ± 45	2.47 ± 0.39	28.0 ± 14.7	341 ± 59	32 (14-55)	96.2
	p	0.000**	0.180	0.176	0.226	0.147	0.000**	0.000**
MGFA	I	145 ± 13	101 ± 35	2.37 (2.16-2.79)	30.1 ± 11.4	336 ± 56	71 ± 49	72.2
at nadir	II	144 ± 14	92 ± 30	2.40 ± 0.38	26.9 ± 8.0	326 ± 62	122 ± 84	51.9
	III	145 ± 17	90 ± 38	2.28 ± 0.44	30.1 ± 13.2	312 ± 65	121 (50-226)	48.6
	IV and V	140 ± 11	98 ± 32	2.10 ± 0.42	35.2 ± 17.8	286 ± 64	152 (74-271)	40.0
	p	0.786	0.489	0.091	0.312	0.103	0.007**	0.117
SGD	No	143 ± 14	95 ± 38	2.39 (2.18-2.71)	28.7 ± 12.1	336 ± 60	80 (34-128)	64.4
	Yes	147 ± 15	94 ± 27	2.14 ± 0.38	30.4 (25.7-36.1)	291 ± 55	144 (74-270)	40.5
	p	0.224	0.903	0.000**	0.246	0.000**	0.000**	0.017*
ePASS	Positive	145 ± 13	95 ± 34	2.45 ± 0.38	29.7 (23.2-36.2)	338 ± 53	81 (32-133)	66.7
	Negative	145 (131-154)	95 ± 34	2.32 ± 0.43	30.3 ± 12.3	315 ± 65	100 (53-175)	52.5
	p	0.566	0.958	0.128	0.557	0.086	0.099	0.182

Note: Numbers are displayed as mean ± standard deviation for variables obeying normal distribution or median with interquartile range in parentheses for variables not obeying normal distribution. * $p < 0.05$, ** $p < 0.01$. ePASS, estimated patient acceptable symptom status; Hb, hemoglobin; ID, iron deficiency; MGFA, Myasthenia Gravis Foundation of America; PostM F, postmenopausal female; PreM F, premenopausal female; SI, serum iron; Tf, transferrin; TIBC, total iron binding capacity; TS, transferrin saturation; SGD, severe generalized disease.

3.2 Factors associated with disease severity at baseline

Disease severity at baseline was represented by SGD and ePASS. Of the 110 patients included, median MG-ADL score was 4 points (IQR 2-7 points), 80 of which had negative ePASS and 37 had SGD. Predictor variables included in the univariate binary logistic regression model were gender, menstruation (premenopausal *versus* postmenopausal female), onset age, interval between onset and immunotherapy, antibody status (MuSK-Ab *versus* others), history of thymoma, thymectomy before inclusion, comorbidity with other autoimmune diseases, hemoglobin level, SI level, Tf level, TIBC level, TS level, TS < 25%, ferritin level, and ID. Predictor variables associated with SGD with a p value < 0.10 were female, positive MuSK-Ab, Tf level, TIBC level, TS < 25%, ferritin level, and ID (Fig. 3a).

Since multicollinearity was evident between Tf and TIBC levels both clinically and statistically, TIBC was excluded from multivariate regression. In multivariate analysis, only ferritin level was independently associated with SGD (Fig. 3b). After adjustment, Tf level or iron deficiency tended to correlate with SGD, but the effect was statistically insignificant.

On the contrary, although univariate analysis yielded some positive results (Fig. 3c), no predictor variables were significantly

associated with negative ePASS in the multivariate analysis.

3.3 Predictors for MMS induction in MG patients first receiving immunotherapy

A total of 106 patients started immunotherapy at inclusion, 3 of which were lost after first visit. The 103 patients remained were followed-up for 6 months and then up to 12 months. Candidate predictors included in the univariate survival analysis were gender (female *versus* male), onset age (< versus ≥ 50 years old), the interval between onset and immunotherapy (< *versus* ≥ 1 year), antibody status (MuSK-Ab *versus* others), history of thymoma, thymectomy before endpoint, comorbidity with other autoimmune diseases, MGFA clinical classification at nadir (ocular MG *versus* others), SGD at baseline, ePASS at baseline, abnormal iron metabolism parameters (below reference range *versus* others, and above reference range *versus* others), ID, oral glucocorticoid exposure, IVMP exposure, TAC exposure, and IVIg exposure.

Among the 103 patients, 49 were female, 7 were positive for MuSK-Ab, 48 were < 50 years old at onset, 50 received first immunotherapy < 1 year after onset, 7 were comorbid with other immune diseases, 17 had histories of thymoma, 20 underwent thymectomy, 32 were categorized as MGFA I, 77 had negative ePASS, and 37 had SGD. As for iron metabolism parameters, SI < 65 μg/dL was observed in 21 of the patients, Tf < 2.00 g/L in 19, TIBC < 250 μg/dL in 11, TS < 25% in 65, ferritin < 24 ng/mL in 11, ferritin > 336 ng/mL in 5, and ID in 56. At 6-month follow-up, 96 of the patients received oral glucocorticoids, 5 IVMP, 6 TAC, 6 IVIg, and 61 reached MMS, with a median follow-up time of 3.53 months (IQR 1.43-6.00 months). At 12-month follow-up, 98 patients remained. Three more patients (9 in total) were exposed to TAC, and 79 reached MMS, with a median follow-up time of 3.53 months (IQR 1.43-6.97 months).

In univariate survival analysis, positive MuSK-Ab, age of onset ≥ 50 years, interval between onset and immunotherapy < 1 year, thymectomy, Tf < 2.00 g/L, and TIBC < 250 μg/dL were predictors potentially associated with MMS induction with a p value < 0.10 at 6-month follow up (Fig. 4a-f). All those predictors except Tf < 2.00 g/L were included in the Cox proportional hazards model, again because of the collinearity between decreased TIBC and decreased Tf. After adjustment, independent predictors for MMS induction were TIBC < 250 μg/dL and the interval between onset and immunotherapy < 1 year (Fig. 4g-h, Table 2). Antibody status, age of onset, and thymectomy were not independent predictors of MMS induction after adjustment (Table 2). TIBC < 250 μg/dL was later replaced by Tf < 2.00 g/L in the Cox regression, but the result was statistically insignificant (HR 1.772, 95% CI 0.917-3.425, $p = 0.089$).

Table 2 Factors potentially associated with the induction of minimal manifestation status at 6-month and 12-month follow-up.

Factors	Follow-up time	Univariate survival analysis HR (95% CI)	*p*	Multivariate survival analysis HR (95% CI)	*p*
Antibody type (MuSK-Ab *versus* others)	6 months	2.691 (0.854-8.484)	0.091	0.778 (0.248-2.443)	0.667
	12 months	N/A	≥ 0.1	N/A	N/A
Age of onset (< 50 years old *versus*	6 months	0.577 (0.347-0.958)	0.034*	1.160 (0.664-2.027)	0.603

≥ 50 years old)	12 months	N/A	≥ 0.1	N/A	N/A
Interval between onset and first	6 months	3.036 (1.663-4.698)	0.000**	2.469 (1.435-4.255)	0.001**
immunotherapy (< *versus* ≥ 1 year)	12 months	2.110 (1.326-3.359)	0.002**	1.812 (1.155-2.841)	0.010*
Thymectomy (post-thymectomy	6 months	0.546 (0.302-0.987)	0.045*	0.580 (0.269-1.250)	0.165
versus others)	12 months	0.614 (0.369-1.021)	0.060	0.691 (0.377-1.268)	0.223
TIBC (< 250 μg/dL *versus* ≥ 250	6 months	6.052 (2.207-16.600)	0.000**	2.707 (1.084-6.757)	0.033*
μg/dL)	12 months	5.376 (2.000-14.450)	0.001**	2.362 (1.206-4.625)	0.012*
TAC exposure (exposed *versus* not	6 months	N/A	≥ 0.1	N/A	N/A
exposed)	12 months	0.519 (0.284-0.950)	0.033*	0.511 (0.218-1.198)	0.123

* $p < 0.05$, ** $p < 0.01$. CI, confidence interval; HR, hazard ratio; MuSK-Ab, anti-muscle-specific tyrosine kinase antibody; N/A, not applicable; TAC, tacrolimus; TIBC, total iron binding capacity.

At 12-month follow-up, the interval between onset and immunotherapy < 1 year, thymectomy, Tf < 2.00 g/L, TIBC < 250 μg/dL, and TAC exposure were predictors potentially associated with MMS with a p value < 0.10 in the uniivariate survival analysis (Fig. 5a-e). Multivariate analysis showed that TIBC < 250 μg/dL and the interval between onset and immunotherapy < 1 year were still independent predictors for MMS (Fig. 5f-g, Table 2). Tf < 2.00 g/L (HR 1.783, 95% CI 1.041-3.056, $p = 0.035$) was also an independent predictor for MMS if TIBC < 250 μg/dL was replaced by it in multivariate analysis (Fig. 5h).

To test the consistence of our results, sensitivity analyses were later performed, in which the 3 lost patients were included in survival analyses at 6-month follow-up, and the 3 lost patients as well as the 5 patients with insufficient follow-up time were included in survival analyses at 12-month follow-up. We contacted the 8 patients through telephone for details on their treatment, and concluded that 6 of them were exposed to oral glucocorticoids, 2 of them took TAC but did not reach our criteria for TAC exposure, and none of them received other kinds of immunotherapy. As our previous results demonstrated that patients with TIBC < 250 μg/dL had more favorable outcome, we hypothesized that the newly-included patients in the sensitivity analyses, who all had TIBC ≥ 250 μg/dL, reached MMS either 3 weeks after initiation of immunotherapy (for the 3 patients lost since first visit), or at their last visit (for the 5 patients with insufficient follow-up time). It turned out in the sensitivity analyses that, at both 6-month and 12-month follow-up, TIBC < 250 μg/dL and the interval between onset and immunotherapy < 1 year were still independent predictors for MMS induction (Table 3). When replacing TIBC < 250 μg/dL in the multivariate model, Tf < 2.00 g/L was still independent predictor for MMS at 12-month follow-up but not at 6-month follow-up (HR 1.817, 95% CI 0.985-3.353, $p = 0.056$ for 6-month follow-up; HR 1.822, 95% CI 1.066-3.114, $p = 0.028$ for 12-month follow-up).

Table 3 Sensitivity analysis of factors potentially associated with the induction of minimal manifestation status at 6-month and 12-month follow-up.

Factors	Follow-up	Univariate survival analysis		Multivariate survival analysis	

	time	HR (95% CI)	*p*	HR (95% CI)	*p*
Gender (female *versus* male)	6 months	N/A	≥ 0.1	N/A	N/A
	12 months	1.443 (0.939-2.217)	0.094	1.545 (0.996-2.398)	0.052
Age of onset (< 50 years old *versus*	6 months	1.631 (0.994-2.677)	0.053	1.254 (0.730-2.153)	0.412
≥ 50 years old)	12 months	N/A	≥ 0.1	N/A	N/A
Interval between onset and first	6 months	2.791 (1.669-4.668)	0.000**	2.165 (1.261-3.717)	0.005**
immunotherapy (< *versus* ≥ 1 year)	12 months	2.002 (1.284-3.121)	0.002**	1.908 (1.227-2.967)	0.004**
MGFA clinical classification at nadir	6 months	1.667 (0.958-2.901)	0.07	1.343 (0.772-2.338)	0.296
(MGFA I *versus* others)	12 months	N/A	≥ 0.1	N/A	N/A
Thymectomy (post-thymectomy	6 months	0.579 (0.325-1.034	0.065	0.744 (0.352-1.574)	0.440
versus others)	12 months	0.662 (0.405-1.081)	0.099	0.790 (0.448-1.390)	0.413
TIBC (< 250 μg/dL *versus* ≥ 250	6 months	4.982 (1.879-13.21)	0.001**	2.228 (1.112-4.464)	0.024*
μg/dL)	12 months	4.199 (1.634-10.79)	0.003**	1.950 (1.002-3.798)	0.049*
TAC exposure (exposed *versus* not	6 months	0.478 (0.203-1.128)	0.092	0.369 (0.089-1.531)	0.169
exposed)	12 months	0.506 (0.281-0.901)	0.023*	0.459 (0.197-1.068)	0.071

Note: For sensitivity analysis at 6-month follow-up, the 3 patients lost since first visit were included; for sensitivity analysis at 12-month follow-up, the 3 lost patients and the 5 patients with insufficient follow-up time were included. None of the 8 patients have decreased Tf and TIBC levels, and were thus considered as MMS at last follow-up (for the 5 patients with insufficient follow-up time) or at 3 weeks after inclusion (for the 3 patients lost since first visit). * $p<0.05$, ** $p<0.01$. CI, confidence interval; HR, hazard ratio; MGFA, Myasthenia Gravis Foundation of America; N/A, not applicable; TAC, tacrolimus; Tf, transferrin; TIBC, total iron binding capacity.

4 Discussion

In this research, we hypothesized that iron metabolism parameters were associated with disease severity and clinical outcome in MG patients receiving first immunotherapy. Key results were: 1) higher ferritin level was independently correlated with SGD but not negative ePASS; 2) TIBC < 250 μg/dL and the interval between onset and immunotherapy < 1 year were independent predictors for MMS at 6-month and 12-month follow-up; 3) Tf < 2.00 g/L was independent predictor for MMS at 12-month but not 6-month follow-up.

4.1 Ferritin and disease severity

MG-ADL is a kind of MG-specific patient-reported outcome measure, which can reflect disease status of MG patients over time (Wolfe et al. , 1999). An increasing number of researches used MG-ADL and variables derived from it for measuring disease severity in MG (Aguirre et al. , 2020, Petersson, Feresiadou, 2021). A previous study showed that MG was more severe in patients > 50 years old, with positive MuSK-Ab, and with thymoma, but the authors did not perform multivariate analysis (Sinaei

et al. , 2022). Indeed, in univariate analysis, we found that patients with MuSK-Ab and an onset age > 50 years old tended to have SGD, but none of them had significant effect after adjustment. Thymoma was not found to be associated with SGD in our patients, in line with a study showing equal MG severity in patients with and without thymoma (Romi et al. , 2003), and possibly because 18 of 19 patients with thymoma in our sample had undergone thymectomy at inclusion.

The only predictor variable significantly associated with SGD after adjustment in our study was serum ferritin (Fig. 3b). Serum ferritin is known to correlate with disease severity in multiple autoimmune diseases including multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis and many others, presumably due to leakage of ferritin from damaged cells leaving unliganded iron somewhere to cause further cellular damage (Kell and Pretorius, 2014). We seemed to be the first to reveal that ferritin level was independently associated with MG severity. Intuitively speaking, our results suggested that, for every 1 ng/mL increase in ferritin, the risk of SGD in non-anemic immunotherapy-naïve MG patients would increase by 1.1%, although it might be oversimplified to automatically assume a linear relation. Additionally, after adjustment, patients with iron deficiency tended to have SDG (Fig. 3b), hinting that correcting iron deficiency might be promising to reduce severity of MG.

Notably, our study showed that ferritin level were not significantly associated with negative ePASS, indicating that ferritin level might be a marker for severe symptoms (SGD), but was not good at distinguishing moderate or worse symptoms (negative ePASS) from mild ones (positive ePASS).

4.2 TIBC, Transferrin and MMS induction

Proposed by MGFA (Jaretzki, Barohn, 2000), MMS was widely adopted as an outcome variable in clinical studies (Akaishi et al. , 2016, Andersen et al. , 2016, Ariatti et al. , 2014, Kanai et al. , 2017, Muppidi et al. , 2019) and a treatment target in clinical practice (Murai et al. , 2018, Sanders et al. , 2016). Previous studies have identified factors facilitating MMS, including age at onset > 50 years (Andersen, Gilhus, 2016), isolated ocular muscle involvement (Li et al. , 2022), and baseline quantitative myasthenia gravis score ≤ 16 points (Li, Yang, 2022). In univariate survival analysis at 6 months, we did find that onset age > 50 years was significantly associated with MMS, although the effect faded out after multivariate analysis. Different from previous studies, ocular MG and milder baseline disease severity were not predictors for MMS induction in our study, possibly arising from different follow-up time and eligibility criteria between our study and previous ones. A previous study has demonstrated that prompt diagnosis (time of diagnosis from onset < 1 year) led to significantly better remission (Mao et al. , 2010). We further revealed that prompt immunotherapy (the interval between onset and immunotherapy < 1 year) was a consistent predictor for MMS at both 6-month and 12-month follow-up. Taken together, our study and the previous one by Mao et al. suggested that timely diagnosis and immunotherapy were essential for MMS. In line with previous results (Mao, Mo, 2010), gender was also not a predictor for MMS in our study.

Since iron predominantly binds to Tf in plasma, TIBC depends on the concentration of Tf (Kasvosve and Delanghe, 2002). In our study, patients with TIBC < 250 μg/dL were more than twice as likely to achieve MMS at 6 months and 12 months after

initiation of immunotherapy (Table 2), and Tf < 2.00 g/L was an independent predictor for MMS at 12-month follow-up, which suggested that for those with TIBC ≥ 250 μg/dL or Tf ≥ 2.00 g/L, more intensive treatment might be warranted. Different from our results, previous studies showed that low TIBC or Tf usually correlated with unfavorable prognosis in patients with inflammatory diseases (Atkinson et al. , 2020, Lv et al. , 2021). We did find that lower TIBC and Tf were associated with SGD in the cross-sectional part of our research (Fig. 3b), but maybe the severe disease status at inclusion were in contrast conducive to MMS induction in the long term after initiation of immunotherapy. Underlying mechanisms of this paradoxical phenomenon needs further exploration in the future.

Interestingly, factors associated with disease severity and MMS induction were different, which could be referred to as a "dissociation" of disease severity and prognosis. This dissociation suggested that patients with milder symptoms at first might not reach MMS earlier in the end, and thus comprehensive management should be carried out for MG patients to not only lessen symptom severity but also improve long-term prognosis. In addition, the dissociation also hinted that ferritin and TIBC/Tf might mediate different inflammatory pathways in the pathogenesis of MG.

4.3 Limitations and highlights

The limitation of this research lies in its relatively small sample size, which might only be able to detect relatively evident effects. Aditionally, since soluble transferrin receptor (sTfR) and sTfR-ferritin index were only tested in a small number of included patients, their effects on SGD, ePASS and MMS induction were not analyzed. Future multicenter prospective studies with sufficient size are needed to further explore the relationship between iron metabolism parameters and disease severity, exacerbation, remission, and recurrence of MG.

Our research has several strengths: 1) novelty: it was probably the first research to analyze the association of iron metabolism parameters with MG severity and outcome; 2) multiple efforts to reduce biases: we examined 2 MG-ADL-derived variables in the cross-sectional analysis, 2 follow-up time in the survival analysis, and performed sensitivity analysis to tackle selection bias, common in cohort studies.

5 Conclusion

Higher ferritin level was independently associated with higher risk of SGD but not negative ePASS in non-anemic immunotherapy-naïve MG patients. TIBC < 250 μg/dL and the interval between onset and immunotherapy < 1 year were independent predictors for better MMS induction at both 6-month and 12-month follow-up after initiation of immunotherapy. Tf < 2.00 g/L was an independent predictor for MMS at 12-month follow-up. Iron metabolism parameters might be promising for evaluating disease severity, guiding clinical decision, and even serving as therapeutic targets in MG patients.

Competing Interests

All the authors report no disclosures or conflicts of interest related to this article.

Author Contributions

Ke Li: conceptualization (equal), data curation (lead), formal analysis (lead), investigation (lead), methodology (equal), software (lead), visualization (lead), writing – original draft (lead). **Li'an Hou**: data curation (lead), formal analysis (equal), investigation (equal), methodology (equal), resources (equal), validation (equal), writing – review & editing (equal). **Ying Tan**: data curation (equal), formal analysis (equal), investigation (equal), methodology (lead), resources (equal)
validation (equal), writing – review & editing (equal). **Yangyu Huang, Jiayu Shi, Jianhua Han & Jingwen Yan**: data curation (equal), formal analysis (equal), investigation (equal), methodology (equal), resources (equal), validation (equal), writing – review & editing (equal). **Yuzhou Guan**: conceptualization (lead), data curation (lead), formal analysis (equal), funding acquisition (lead), investigation (equal), methodology (equal), project administration (lead), resources (lead), supervision (lead), validation (lead), writing – review & editing (lead).

Data Availability Statement

The anonymized data that support the findings of our study are available from the first or corresponding author on reasonable request and approval by the relevant boards of the corresponding institution.

Funding

This work was supported by the Non-Profit Central Research Institute Fund of Chinese Academy of Medical Sciences (grant number: 2019XK320039).

References

Abo-Krysha N, Rashed L. The role of iron dysregulation in the pathogenesis of multiple sclerosis: an Egyptian study. Multiple sclerosis (Houndmills, Basingstoke, England). 2008;14:602-8.

Aguirre F, Manin A, Fernandez VC, Justo ME, Leoni J, Paz ML, et al. C3, C5a and anti-acetylcholine receptor antibody as severity biomarkers in myasthenia gravis. Therapeutic advances in neurological disorders. 2020;13:1756286420935697.

Akaishi T, Suzuki Y, Imai T, Tsuda E, Minami N, Nagane Y, et al. Response to treatment of myasthenia gravis according to clinical subtype. BMC Neurol. 2016;16:225.

Andersen JB, Gilhus NE, Sanders DB. Factors affecting outcome in myasthenia gravis. Muscle & nerve. 2016;54:1041-9.

Ariatti A, Stefani M, Miceli P, Benuzzi F, Galassi G. Prognostic factors and health-related quality of life in ocular Myasthenia Gravis (OMG). The International journal of neuroscience. 2014;124:427-35.

Atkinson SR, Hamesch K, Spivak I, Guldiken N, Cabezas J, Argemi J, et al. Serum Transferrin Is an Independent Predictor of Mortality in Severe Alcoholic Hepatitis. The American journal of gastroenterology. 2020;115:398-405.

Carr AS, Cardwell CR, McCarron PO, McConville J. A systematic review of population based epidemiological studies in Myasthenia Gravis. BMC Neurol. 2010;10:46.

Cronin SJF, Woolf CJ, Weiss G, Penninger JM. The Role of Iron Regulation in Immunometabolism and Immune-Related Disease. Frontiers in molecular biosciences. 2019;6:116.

Evoli A, Antonini G, Antozzi C, DiMuzio A, Habetswallner F, Iani C, et al. Italian recommendations for the diagnosis and treatment of myasthenia gravis. Neurol Sci. 2019;40:1111-24.

Gono T, Kawaguchi Y, Hara M, Masuda I, Katsumata Y, Shinozaki M, et al. Increased ferritin predicts development and severity of acute interstitial lung disease as a complication of dermatomyositis. Rheumatology (Oxford, England). 2010;49:1354-60.

Jaretzki A, 3rd, Barohn RJ, Ernstoff RM, Kaminski HJ, Keesey JC, Penn AS, et al. Myasthenia gravis: recommendations for clinical research standards. Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America. Neurology. 2000;55:16-23.

Kanai T, Uzawa A, Kawaguchi N, Himuro K, Oda F, Ozawa Y, et al. Adequate tacrolimus concentration for myasthenia gravis treatment. European journal of neurology. 2017;24:270-5.

Kasvosve I, Delanghe J. Total iron binding capacity and transferrin concentration in the assessment of iron status. Clinical chemistry and laboratory medicine. 2002;40:1014-8.

Kawasumi H, Gono T, Kawaguchi Y, Kaneko H, Katsumata Y, Hanaoka M, et al. IL-6, IL-8, and IL-10 are associated with hyperferritinemia in rapidly progressive interstitial lung disease with polymyositis/dermatomyositis. Biomed Res Int. 2014;2014:815245.

Kell DB, Pretorius E. Serum ferritin is an important inflammatory disease marker, as it is mainly a leakage product from damaged cells. Metallomics : integrated biometal science. 2014;6:748-73.

Kobayashi S, Shoda H, Seri Y, Nagafuchi Y, Sasaki O, Kubo K, et al. Characteristics of antisynthetase syndrome patients with hyperferritinaemia. Scandinavian journal of rheumatology. 2017;46:161-3.

Li Y, Yang S, Dong X, Li Z, Peng Y, Jin W, et al. Factors affecting minimal manifestation status induction in myasthenia gravis. Therapeutic advances in neurological disorders. 2022;15:17562864221080520.

Lv Y, Chen L, Liang X, Liu X, Gao M, Wang Q, et al. Association between iron status and the risk of adverse outcomes in COVID-19. Clinical nutrition (Edinburgh, Scotland). 2021;40:3462-9.

Mao ZF, Mo XA, Qin C, Lai YR, Olde Hartman TC. Course and prognosis of myasthenia gravis: a systematic review. European journal of neurology. 2010;17:913-21.

McDonagh TA, Metra M, Adamo M, Gardner RS, Baumbach A, Böhm M, et al. Corrigendum to: 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: Developed by the Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) With the special contribution of the Heart Failure Association (HFA) of the ESC. European heart journal. 2021;42:4901.

Mendoza M, Tran C, Bril V, Katzberg HD, Barnett C. Patient-acceptable symptom states in myasthenia gravis. Neurology. 2020;95:e1617-e28.

Muppidi S, Utsugisawa K, Benatar M, Murai H, Barohn RJ, Illa I, et al. Long-term safety and efficacy of eculizumab in generalized

myasthenia gravis. Muscle & nerve. 2019;60:14-24.

Murai H, Utsugisawa K, Nagane Y, Suzuki S, Imai T, Motomura M. Rationale for the clinical guidelines for myasthenia gravis in Japan. Annals of the New York Academy of Sciences. 2018;1413:35-40.

Nairz M, Weiss G. Iron in infection and immunity. Molecular aspects of medicine. 2020;75:100864.

Petersson M, Feresiadou A, Jons D, Ilinca A, Lundin F, Johansson R, et al. Patient-Reported Symptom Severity in a Nationwide Myasthenia Gravis Cohort: Cross-sectional Analysis of the Swedish GEMG Study. Neurology. 2021;97:e1382-91.

Romi F, Gilhus NE, Varhaug JE, Myking A, Aarli JA. Disease severity and outcome in thymoma myasthenia gravis: a long-term observation study. European journal of neurology. 2003;10:701-6.

Sanders DB, Wolfe GI, Benatar M, Evoli A, Gilhus NE, Illa I, et al. International consensus guidance for management of myasthenia gravis: Executive summary. Neurology. 2016;87:419-25.

Sfagos C, Makis AC, Chaidos A, Hatzimichael EC, Dalamaga A, Kosma K, et al. Serum ferritin, transferrin and soluble transferrin receptor levels in multiple sclerosis patients. Multiple sclerosis (Houndmills, Basingstoke, England). 2005;11:272-5.

Sinaei F, Fatehi F, Oveis Gharan S, Ehsan S, Kamali K, Nafissi S. Disease severity and response to treatment in Iranian patients with myasthenia gravis. Neurol Sci. 2022;43:1233-7.

Torti SV, Torti FM. Iron and cancer: more ore to be mined. Nat Rev Cancer. 2013;13:342-55.

van Rensburg SJ, Kotze MJ, Hon D, Haug P, Kuyler J, Hendricks M, et al. Iron and the folate-vitamin B12-methylation pathway in multiple sclerosis. Metab Brain Dis. 2006;21:117-33.

von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. Lancet (London, England). 2007;370:1453-7.

Wolfe GI, Herbelin L, Nations SP, Foster B, Bryan WW, Barohn RJ. Myasthenia gravis activities of daily living profile. Neurology. 1999;52:1487-9.

Figure and Table Captions

Fig. 1 Flowchart demonstrating the process of the research. ePASS, estimated patient acceptable symptom status; MG, myasthenia gravis; MMS, minimal manifestation status; SGD, severe generalized disease

Fig. 2 Comparison of ferritin levels in different subgroups of myasthenia gravis patients. Bars are median concentration of serum ferritin and whiskers are interquartile range. * $p<0.05$, ** $p<0.01$. ePASS, estimated patient acceptable symptom status; MGFA, Myasthenia Gravis Foundation of America; ns, not significant; PostM, postmenopausal; PreM, premenopausal; SGD, severe generalized disease

Fig. 3 Factors associated with SGD and negative ePASS. (a) Female sex, positive MuSK-Ab, Tf level, TIBC level, TS < 25%, ID, and ferritin level were potentially associated with SGD with a p value < 0.10 in univariate binary logistic regression; (b) Ferritin level was the only included predictor variables independently associated with SGD in the multivariate logistic regression model; (c) Candidate predictor variables which might be associated with negative ePASS in the univariate binary logistic regression model, none of which was independently associated with negative ePASS in multivariate analysis. $^{*}p<0.05$, $^{**}p<0.01$. CI, confidence interval; ePASS, estimated patient acceptable symptom status; Fer, ferritin; ID, iron deficiency; MuSK-Ab, anti-muscle-specific tyrosine kinase antibody; OR, odds ratio; Tf, transferrin; TIBC, total iron binding capacity; TS, transferrin saturation; SGD, severe generalized disease

Fig. 4 Kaplan-Meier curves and Cox regression for candidate predictors of MMS induction at 6-month follow-up. (a-f) Positive MuSK-Ab, age of onset ≥ 50 years old, interval between onset and immunotherapy < 1 year, thymectomy, Tf < 2.00 g/L, and TIBC < 250 μg/dL were predictors potentially associated with MMS induction with a p value < 0.10 at 6-month follow-up in univariate survival analysis, as shown by the Kaplan-Meier curves. (g-h) Cox proportional hazards model showed that TIBC < 250 μg/dL ($p = 0.033$) and the interval between onset and immunotherapy < 1 year ($p = 0.001$) were independent predictors for MMS. MMS, minimal manifestation status; MuSK-Ab, anti-muscle-specific tyrosine kinase antibody; Tf, transferrin; TIBC, total iron binding capacity

Fig. 5 Kaplan-Meier curves and Cox regression for candidate predictors of MMS induction at 12-month follow-up. (a-e) Interval between onset and immunotherapy < 1 year, thymectomy, Tf < 2.00 g/L, TIBC < 250 μg/dL, and TAC exposure were predictors potentially associated with MMS induction with a p value < 0.10 at 12-month follow-up in univariate survival analysis, as shown by the Kaplan-Meier curves. (f-g) Cox proportional hazards model showed that TIBC < 250 μg/dL ($p = 0.012$) and the interval between onset and immunotherapy < 1 year ($p = 0.010$) were independent predictors for MMS. (h) Tf < 2.00 g/L ($p = 0.035$) was also an independent predictor for MMS if TIBC < 250 μg/dL was replaced by it in Cox regression. MMS, minimal manifestation status; TAC, tacrolimus; Tf, transferrin; TIBC, total iron binding capacity

Table 1 Iron metabolism parameters in MG patients

Note: Numbers are displayed as mean ± standard deviation for variables obeying normal distribution or median with interquartile

range in parentheses for variables not obeying normal distribution

* $p<0.05$, ** $p<0.01$. ePASS, estimated patient acceptable symptom status; Hb, hemoglobin; ID, iron deficiency; MGFA, Myasthenia Gravis Foundation of America; PostM F, postmenopausal female; PreM F, premenopausal female; SI, serum iron; Tf, transferrin; TIBC, total iron binding capacity; TS, transferrin saturation; SGD, severe generalized disease

Table 2 Factors potentially associated with the induction of minimal manifestation status at 6-month and 12-month follow-up

* $p<0.05$, ** $p<0.01$. CI, confidence interval; HR, hazard ratio; MuSK-Ab, anti-muscle-specific tyrosine kinase antibody; N/A, not applicable; TAC, tacrolimus; Tf, transferrin; TIBC, total iron binding capacity

Table 3 Sensitivity analysis of factors potentially associated with the induction of minimal manifestation status at 6-month and 12-month follow-up

Note: For sensitivity analysis at 6-month follow-up, the 3 patients lost since first visit were included; for sensitivity analysis at 12-month follow-up, the 3 lost patients and the 5 patients with insufficient follow-up time were included. None of the 8 patients have decreased Tf and TIBC levels, and were thus considered as MMS at last follow-up (for the 5 patients with insufficient follow-up time) or at 3 weeks after inclusion (for the 3 patients lost since first visit)

* $p<0.05$, ** $p<0.01$. CI, confidence interval; HR, hazard ratio; MGFA, Myasthenia Gravis Foundation of America; MuSK-Ab, anti-muscle-specific tyrosine kinase antibody; N/A, not applicable; TAC, tacrolimus; Tf, transferrin; TIBC, total iron binding capacity