

# HAPTOGLOBIN STUDY IN MYASTHENIA GRAVIS

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**Abstract – Objective:** A cross-sectional study of haptoglobin (Hp) in myasthenia gravis (MG) was designed, with the objective to identify its values and correlate them with different disease status. **Method:** 46 patients were enrolled in the study, all having disease severity established according to the quantitative myasthenia gravis strength scores (QMGSS). Based on the functional scale determined by Myasthenia Gravis Foundation of America (MGFA) recommendations, patients were classified as having: complete stable remission (CSR; n=10); minimal manifestations–0 (MM0; n=6), minimal manifestations–1 (MM1; n=4); pharmacological remission (PR; n=6). Two other groups participated: thymomatous patients (T; n=10) and patients without immunosuppression or thymectomy, until the assessment for Hp (WIT; n=10). Hp dosage was done by immunonephelometry, blindly to clinical data. Student's t-test, Anova test and linear regression were employed for statistical analyses. **Results:** Statistically significant differences occurred between CSR+MM0xWIT groups (86.62x157.57, p<0.001) and PR+MM1xWIT groups (73.93x157.57, p<0.001). Linear regression showed correlation between Hp levels and QMGSS (r=0.759, p<0.001). **Conclusion:** Our results suggest that Hp may be useful in clinical practice as a disease severity marker in MG.

KEY WORDS: haptoglobin, acute phase response, myasthenia gravis, immune disorders.

## Estudo sobre a haptoglobina na miastenia grave

**Resumo – Objetivo:** Desenhou-se estudo transversal sobre a haptoglobina (Hp) na miastenia grave (MG) com o objetivo de identificar seus valores e correlacioná-los a diferentes condições na doença. **Método:** 46 pacientes foram incluídos, todos tendo a gravidade da doença estabelecida segundo escores internacionais (QMGSS). Os pacientes tiveram seu estado funcional determinado de acordo com a Myasthenia Gravis Foundation of America (MGFA) e classificados em: remissão completa estável (CSR; n=10); mínima manifestação–0 (MM0; n=6), mínima manifestação–1 (MM1; n=4); remissão farmacológica (PR; n=6). Dois outros grupos participaram: pacientes timomatosos (T; n=10) e pacientes sem imunossupressão ou timectomia, até o momento da inclusão no estudo (WIT; n=10). A dosagem de Hp foi realizada por imunonefelometria, de modo cego quanto à clínica. As análises estatísticas incluíram o teste de Student, Anova e regressão linear. **Resultados:** Observou-se diferença significativa entre os grupos CSR+MM0xWIT (86,62x157,57, p<0,001) e entre PR+MM1xWIT (73,93x157,57, p<0,001). A regressão linear mostrou correlação positiva entre os valores de Hp e os escores QMGSS (r=0,759, p<0,001). **Conclusão:** O estudo sugere que valores altos de Hp se correlacionaram a maior gravidade da MG.

PALAVRAS-CHAVE: haptoglobina, resposta de fase aguda, miastenia grave, doença autoimune.

Haptoglobin (Hp) is an acute phase inflammatory  $\alpha$ 2-glycoprotein synthesized in the liver<sup>1,2</sup>. It binds free hemoglobin in the plasma and thereby inhibits its oxidative activity in other tissues<sup>1-3</sup>. In the immune system, Hp modulates the function of T helper lymphocytes and inhibits the CD22 binding to activated TNF- $\alpha$ <sup>3</sup>. Due to polymorphisms in the gene encoding Hp on chromosome 16q22, there are three distinct human Hp phenotypes: Hp 1-1, Hp 2-1 and Hp 2-2<sup>1,3,4</sup>. Hp has been used as a diagnostic and

prognostic marker in several neurological and non-neurological conditions<sup>1</sup>. Hp 1-1 phenotype has been associated with better seizure control in some epileptic syndromes<sup>5,6</sup>. A decreased risk of vasospasm after aneurismal subarachnoid hemorrhage has been associated with Hp 1-1<sup>7</sup>. In Guillain-Barré syndrome (GBS) high levels of serum Hp were identified and attributed to the concomitant raise in Interleukin 6 (IL 6)<sup>8</sup>. More recently, elevation of Hp levels in the cerebrospinal fluid (CSF) of GBS patients was reported<sup>9</sup>.

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Received 18 September 2007, received in final form 13 December 2007. Accepted 12 February 2008.

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Myasthenia gravis (MG) is the most frequent disorder of the neuromuscular junction and is characterized by fluctuating weakness either restricted to extrinsic ocular muscles or expressed in a more generalized distribution<sup>10,11</sup>. It is an autoimmune disease caused by antibodies against acetylcholine receptors (Ab-AChR) in about 95% of cases, and may be associated with other autoimmune diseases<sup>10</sup>. T lymphocytes and proinflammatory cytokines play key roles in the pathogenesis of MG<sup>10,12</sup>. Thymus is involved in the disease process since thymic hyperplasia occurs in 60% of MG cases and thymoma in 15%<sup>10,11</sup>.

As MG shares some pathogenic features with autoimmune diseases, such as GBS<sup>8,10</sup>, in which Hp was proven useful, we investigated Hp values in MG patients looking for its possible utility as a marker of disease activity.

## METHOD

This study was approved by the Ethics Committee of Faculty of Medical Sciences, Clinical Hospital, Campinas State University. A written informed consent was obtained from all patients.

### Study design

In a cohort of prospectively selected MG patients, we performed a cross-sectional study of Hp values.

### Subjects selection

Adult patients fulfilling clinical and laboratorial criteria for MG and attending the Neuromuscular outpatient clinic were enrolled in the study. Quantitative myasthenia gravis strength score (QMGSS)<sup>13</sup> was employed to assess disease severity. Patient's functional status was determined in accordance to Myasthenia Gravis Foundation of America (MGFA) recommendations<sup>13</sup> and divided into four functional groups: 1) *Complete stable remission* (CSR): no symptoms or signs of MG (clinical score=0) for at least one year and without immunosuppressive therapy during that time; 2) *Minimal manifestations – 0* (MM0): no symptoms or functional limitations due to MG, but patient had weakness of some muscles upon examination (clinical score=1) without immunosuppressive therapy for at least one year; 3) *Pharmacologic remission* (PR), the same criteria as for CSR, except that patient continued to take immunosuppressive therapy; 4) *Minimal manifestations – 1* (MM1), the same criteria as for MM-0, except that patient continued to take immunosuppressive therapy. Two other groups of patients were included, also evaluated by QMGSS: 5) *Thymomatous* (T) patients with histopathological diagnosis, regardless of symptoms or treatment in the moment of the study; 6) MG symptomatic patients (QMGSS>1) *without immunosuppression or thymectomy* (WIT). Patients in the WIT group were referred to our institution, eventually using anticholinesterasic drugs, and they were submitted to immunosuppressive treatment after venous puncture for Hp study.

### Haptoglobin dosage

Blood samples from each patient were collected and the Hp

concentration was determined by immunonephelometry (Dade Behring, Marburg, Germany), blindly to the classification of patients in the study.

### Statistical analyses

Differences between two groups were compared by Student's t-test. Comparison of Hp levels in multiple groups was accomplished by the Anova test and Tukey's post-hoc analysis. We employed linear regression to analyze correlations between Hp levels and QMGSS. MG patients with other active immune-mediated co-morbidity were excluded from final analysis. Analyses were performed on SYSTAT 10.2 software and the level of significance was assumed at  $p < 0.05$ .

## RESULTS

Forty-six patients participated in the study, 27 women and 19 men. Mean age was 41.7 years (range 18 to 76 years) and the mean duration of disease was 7.1 years. There were 10 patients in the CSR group, 6 in MM-0, 6 in PR, 4 in MM-1, 10 in T and 10 in WIT group. For statistical analyses, groups CSR and MM0 were fused into final fCSR group. Similarly, groups PR and MM1 were fused into final fPR group. Clinical and laboratorial data of patients on group fCSR, fPR, T and WIT are displayed on Tables 1 to 4.

In Tables 1 and 2 we can observe three patients with active autoimmune co-morbidities, indicated by clinical history through chart revision, previous specialized clinical and laboratory follow-up and specific treatment in course. They were cases 7 and 13 in the group CSR and case 22 in the group PR; their Hp values were the highest

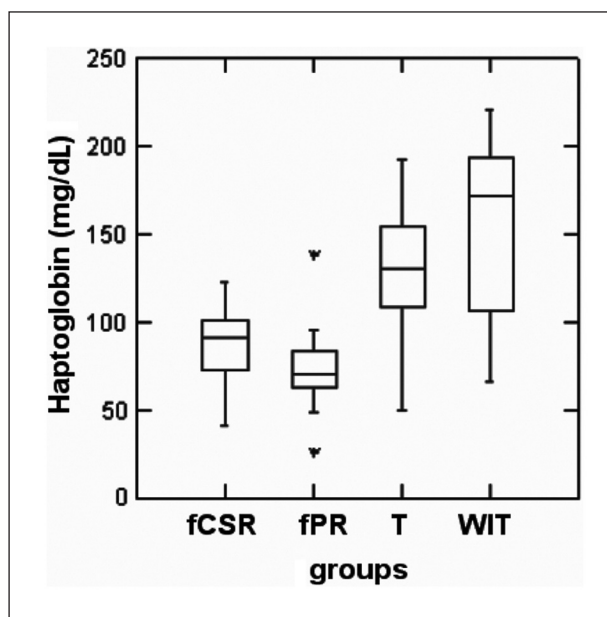


Fig 1. Box-and-whiskers plot showing Hp levels in the different groups of MG patients. The box extends from the 25<sup>th</sup> percentile to the 75<sup>th</sup> percentile, with a horizontal line at the median (50<sup>th</sup> percentile). Whiskers extend down to the smallest value and up to the largest.

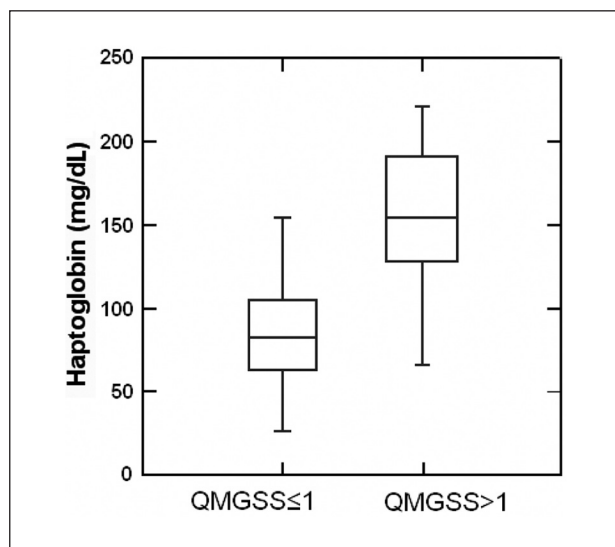


Fig 2. Box-and-whiskers plot showing Hp levels in asymptomatic (QMGSS $\leq$ 1) and symptomatic (QMGSS $>$ 1) MG patients. The box extends from the 25<sup>th</sup> percentile to the 75<sup>th</sup> percentile, with a horizontal line at the median (50<sup>th</sup> percentile). Whiskers extend down to the smallest value and up to the largest.

in each respective group so these patients were excluded from final analysis to avoid a statistical bias.

Figure 1 shows Hp levels in the different groups of MG patients. The mean Hp value in fCSR, fPR, WIT and T groups were 86.62, 73.93, 157.57 and 126.39 mg/dL, respectively. Comparing Hp values in these different groups (Fig

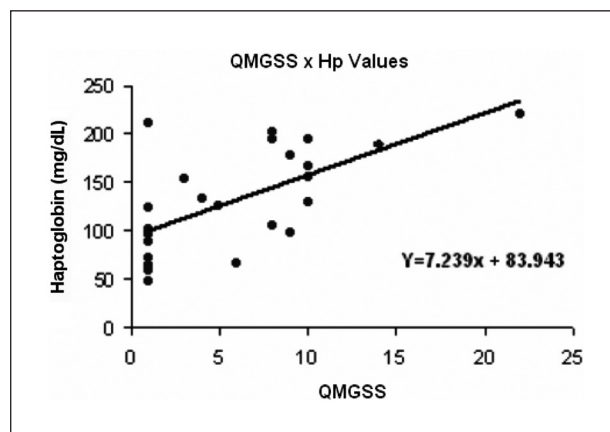


Fig 3. Linear regression analysis of Hp values and QMGSS in MG patients.

1), we found statistically significant differences only between groups fCSRxWIT (86.62x157.57,  $p<0.001$ ) and between groups fPRxWIT (73.93x157.57,  $p<0.001$ ).

We hypothesized that Hp levels correlated with disease severity in MG patients and performed two additional analyses. First, we compared Hp in symptomatic (QMGSS $>$ 1) versus asymptomatic (QMGSS $\leq$ 1) patients (regardless of medication status) and found significantly lower results in the latter group (154.1x95.5,  $p<0.001$  – Fig 2). Second, we used linear regression analyses that showed a significant correlation between Hp levels and QMGSS ( $r=0.759$ ,  $p<0.001$  – Fig 3).

Table 1. Hp levels on fCSR (CSR+MM1) group.

n <sup>o</sup>	Sex	Ao / As (years)	Autoimmune comorbidity	Thymectomy	Thymus histopathology	Immune Therapy	QMGSS Score	Hp value
1	F	26 / 34	No	Yes	Involution	No	00	75.3
2	F	30 / 42	No	Yes	Hyperplasia	No	00	95.9
3	F	21 / 31	No	Yes	Hyperplasia	No	00	40.9
4	F	40 / 64	No	Yes	Normal	No	00	73.3
5	F	39 / 59	No	Yes	Hyperplasia	No	00	55.3
6	F	15 / 22	No	Yes	Hyperplasia	No	00	78.3
7	F	32 / 53	Hashimoto*	No	–	No	00	160.0
8	F	19 / 28	No	Yes	Hyperplasia	No	00	110.0
9	M	23 / 36	Hashimoto <sup>#</sup>	Yes	Hyperplasia	No	00	92.8
10	M	28 / 35	No	No	–	No	00	119.0
11	F	14 / 25	No	Yes	Hyperplasia	No	01	89.7
12	F	28 / 39	No	Yes	Normal	No	01	59.2
13	F	27 / 33	IUR*	Yes	Hypertrofia	No	01	211.0
14	M	22 / 27	No	Yes	Hyperplasia	No	01	99.0
15	F	32 / 39	No	Yes	Hyperplasia	No	01	123.0
16	M	50 / 62	No	Yes	Atrophy	No	01	101.0

F, female; M, male; Ao, age of disease onset; As, age at study; \*active illness; <sup>#</sup>illness in remission; IUR, inflammatory ulcerative rectocolitis; Hashimoto, Hashimoto thyroiditis.

Table 2. Hp levels on fPR (PR+MMI) group.

nº	Sex	Ao / As (years)	Autoimmune comorbidity	Thymectomy	Thymus histopathology	Immune therapy	QMGSS score	Hp value
17	M	20 / 23	No	No	–	Aza / Pred	00	139.0
18	F	61 / 66	No	No	–	Pred	00	83.8
19	M	62 / 68	No	No	–	Aza	00	72.5
20	M	64 / 72	No	No	–	Aza	00	63.7
21	F	18 / 22	No	No	–	Aza	00	26.7
22	M	27 / 35	Pemphigus	Yes	Hyperplasia	Aza / Pred	00	187.0
23	M	33 / 44	IUR#	Yes	Atrophy	Pred	01	63.4
24	F	48 / 51	No	No	–	Aza	01	49.2
25	F	32 / 34	No	No	–	Aza / Pred	01	96.0
26	M	24 / 26	No	No	–	Aza	01	71.1

F, female; M, male; Ao, age of disease onset; As, age at study; \*active illness; #illness in remission; IUR, inflammatory ulcerative rectocolitis; Aza, azatioprine; Pred, prednisone.

Table 3. Hp levels on T group.

nº	Sex	Ao / As years	Autoimmune comorbidity	Thymus surgery	Thymus histology	Immune therapy	Score QMGSS	Hp value
27	M	44 / 55	No	Yes	Lymphoepithelial T	No	05	126.0
28	F	52 / 57	No	Yes	Invasive T	No	04	133.0
29	F	46 / 56	No	Yes	No invasive T	Aza	00	109.0
30	F	32 / 46	No	Yes	Multifocus T	No	00	82.1
31	M	17 / 25	No	Yes	Invasive T	Aza	10	130.0
32	M	33 / 37	No	Yes	Invasive T	Pred	10	155.0
33	M	26 / 39	No	Yes	No invasive T	Aza	00	131.0
34	M	19 / 22	No	Yes	Invasive T	Aza	10	193.0
35	M	33 / 37	No	Yes	Lymphoepithelial T	Pred	00	155.0
36	F	26 / 37	No	Yes	Microthymoma	No	00	49.8

F, female; M, male; Ao, age of disease onset; As, age at study; Aza, azatioprine; Pred, prednisone; T, thymoma.

## DISCUSSION

To our knowledge, this is the first study of Hp values in patients with MG. We found that groups fCSR and fPR presented lower Hp levels when compared to WIT group, composed of MG patients in their natural disease state, eventually taking symptomatic drugs, but without effective treatment. Similarly, symptomatic patients had higher Hp levels in comparison to asymptomatic patients. Group T did not present significantly different Hp levels in comparison to other groups. This is possibly explained by the clinical heterogeneity of the group, which includes five patients in remission and five patients with active disease. Further, there was a positive correlation between Hp levels and QMGSS among symptomatic MG individuals. Overall, these findings indicate that active disease (QMGSS>1) is associated with raised Hp levels, regardless of thymus status or medication in use.

The production of Ab-AChR is mediated by cytokines produced by CD4+ and CD8+ T helper (Th) cells<sup>12</sup>. IL-4, IL-6

and IL-10, all Th2 cytokines, are an efficient growth promoter for B-cell proliferation and differentiation, acting mainly in disease progression and persistence<sup>12</sup>. Interferon- $\gamma$ , a Th1 cytokine, is important in inducing B-cell maturation, acting more agile at the onset of MG, probably being one of the initiating factors in the induction of the disease<sup>12</sup>. In contrast, transforming growth factor- $\beta$  (TGF- $\beta$ ) and IFN- $\alpha$  exerts immunosuppressive effects, which include the down regulation of both Th1 and Th2 cytokines in MG<sup>12</sup>.

Hp is an acute phase protein that binds free hemoglobin and removes it from the circulation to prevent kidney injury and iron loss following hemolysis, thus having an important antioxidant action<sup>2,3</sup>. In addition, Hp acts as a potent immunosuppressor of lymphocyte function and modulates the Th1 and Th2 balance within the body<sup>1,3</sup>. It also binds to human B-cell by the CD22 surface receptor and is involved in immune and inflammatory responses<sup>3</sup>. CD22 mediates B-cell interactions with erythrocytes, T lymphocytes, monocytes and neutrophils by specific bind-

Table 4. Hp levels on WIT group.

nº	Sex	Ao / As (years)	Autoimmune comorbidity	Thymectomy	Immune therapy	Score QMGSS	Hp value
37	F	34 / 50	No	No	No	03	153.0
38	M	73 / 76	No	No	No	08	194.0
39	F	32 / 34	No	No	No	09	98.5
40	F	27 / 30	No	No	No	08	107.0
41	M	48 / 48	No	No	No	06	66.2
42	M	66 / 66	No	No	No	09	178.0
43	F	31 / 32	No	No	No	22	221.0
44	F	17 / 18	No	No	No	14	189.0
45	M	50 / 60	No	No	No	08	202.0
46	F	22 / 23	No	No	No	10	167.0

F, female; M, male; Ao, age of disease onset; As, age at study.

ing to glycoprotein<sup>3</sup>. When Hp binds to CD22 it inhibits the TNF- $\alpha$ -activated action<sup>3</sup>. TNF- $\alpha$  is a proinflammatory cytokine implicated in MG pathogenesis<sup>14,15</sup>. The soluble recombinant TNF receptor Fc protein, etanercept, has been tested in MG patients showing benefit in those with low plasma IL-6 levels<sup>14</sup>.

The high serum Hp levels observed in the study are consistent with an active inflammatory state in MG patients<sup>13</sup>. Hp is closely related to IL-6, which is the main stimulatory cytokine responsible for its production in the liver<sup>13</sup>. Interestingly, IL-6 acts in MG as an important growth and differentiation factor of B lymphocytes, which are directly related to the damage in the mioneural plate<sup>12</sup>. Moreover, lower IL-6 levels have been associated with better therapeutic results in MG patients treated with etanercept<sup>14</sup>. It is thus expected that in cases of active disease, there is aggression to the mioneural plate and therefore, high levels of IL-6. So, indirectly there would be raised Hp values. This reasoning could also explain the relation between the clinical score and Hp levels, since more intense inflammatory process may be associated with more prominent symptoms<sup>11</sup>. Similarly, there are elevated serum Hp levels in GBS patients, which have been explained by the concomitant increase of IL-6 levels<sup>8</sup>.

Hp values are not disease-specific, explaining that patients in clinical remission of MG, but presenting another active autoimmune illness had high values of Hp than MG patients without active autoimmune comorbidity. In these cases, Hp would reflect the activity of disease comorbidity, not that of MG. According to Christensen et al.<sup>16</sup>, 14% of MG patients presented concomitant autoimmune disease, therefore clinicians must be alert to evaluate each autoimmune illness independently, and consider Hp values in a contextual setting.

In conclusion, we suggest that Hp can be useful as a marker of clinical activity in MG without another associated illness.

**ACKNOWLEDGEMENTS** – The authors wish to express special thanks to Prof. Dr. Konradin Metzke for his assistance with thymus histopathology; to the staff of the Thoracic Surgery Department (Profs. Drs. José Cláudio Seabra, José Geraldo dos Santos, Ivan C Toro and Ricardo M Kalaf).

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