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## Anti-Heat Shock Protein 70 antibody levels are increased in myasthenia gravis and Guillain-Barré syndrome

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### ABSTRACT

Myasthenia gravis (MG) is an autoimmune disorder where patients develop autoantibodies towards skeletal muscle proteins (e.g. acetylcholine receptor and muscle specific kinase), causing weakness in striated muscles. Ocular MG (OMG) represents a subtype of (MG) affecting only the periocular muscles. The pathogenesis of this phenotype remains unclear. Heat Shock Protein 70 (Hsp70) plays a role in immune regulation. Antibodies against this protein are associated with several autoimmune diseases, and its biological significance has been shown *in vivo*. We have therefore examined the concentration of anti-Hsp70 antibodies in sera from 35 OMG patients and 94 patients with generalized MG (GMG) using ELISA assays. The antibody concentrations were compared to those in patients with multiple sclerosis (MS), Guillain-Barré syndrome (GBS) and to healthy controls.

MG patients had significantly higher anti-Hsp70 antibody concentrations than both MS patients and healthy controls. GBS patients had higher antibody levels than all other groups. No difference in antibody levels was found when comparing OMG and GMG. Our results suggest that patients with MG and GBS have a previous or current increased exposure to Hsp70 antigens. The similarity between GMG and OMG strengthens the hypothesis that OMG represents a systemic disease, similar to GMG.

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### 1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder where patients experience weakness in voluntary muscles, including respiratory function. It is in most cases caused by circulating antibodies targeting the nicotinic acetylcholine receptor (AChR) on skeletal muscle cell membranes, but antibodies towards muscle specific kinase (MuSK) can also have pathogenic significance (Pascale Ter Beek et al., 2009; Vincent and Leite, 2005). A subgroup of MG patients experience only periocular motor symptoms such as diplopia and ptosis, and are hence referred to as ocular MG (OMG). The reasons for the purely periocular involvement are unclear. Approximately 40% of OMG patients are anti-AChR antibody positive in conventional assays, (Luchanok and Kaminski, 2008) but the sensitivity is higher in cell culture based assays. However, this has only been demonstrated for GMG sera, and not in sera from OMG patients (Leite et al., 2008).

Antibodies against Hsp70 have been detected in sera from patients with sensory neuronal hearing loss (Tebo et al., 2006), systemic lupus erythematosus (Tasneem et al., 2001), juvenile idiopathic arthritis (Zlacka et al., 2006) and MG (Munakata et al., 2008). These studies have focused on the presence of antibodies, and have not aimed at providing any etiological evidence. When applying anti-Hsp70 antibodies together with an allergy-inducing agent, mice did not develop the allergic dermatitis, but rather gained systemic antigen specific tolerance which could be adoptively transferred (Yusuf et al., 2009).

The Heat Shock Proteins (Hsp) are divided into subfamilies according to weight (Multhoff, 2007). They play a crucial role in functioning as chaperones to prevent protein misfolding and aggregation (Bukau and Horwich, 1998). While many of the HSPs are expressed at low levels in most eukaryotic cells, they can be induced upon cellular stress such as increased temperature, radiation, exposure to various chemicals, oxidative stress and various physiological and pathological stimuli (Multhoff, 2007; Tsan and Gao, 2004). In addition to being chaperone proteins, the HSPs play a part in antigen presentation and cross-presentation, (Li et al., 2002) and

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function as cytokines to induce production of pro-inflammatory cytokines and promote dendritic cell maturation (Wang et al., 2002; Asea et al., 2000).

Hsp70 is believed to be involved in the pathogenesis of several autoimmune disorders, including Behcet's disease (Birtas-Atesoglu et al., 2008), Grave's disease (Ratanachaiyavong et al., 1991; Hunt et al., 2001) and multiple sclerosis (MS) (Salveti et al., 1996). Its ability to augment antigen presentation has been shown in experimentally induced diabetes mellitus (Millar et al., 2003), and increased levels are found locally in experimental autoimmune neuritis, a Guillain-Barré syndrome (GBS) model (Zhang et al., 2009).

Hsp70 therefore plays an important role in antigen presentation and development of tolerance, and antibody-mediated interference of its function can alter the immune response for antigens being presented by antigen presenting cells. In this study we investigated whether patients with generalized and ocular MG had altered anti-Hsp70 levels, as compared to healthy controls and patients with the widespread immune disorders MS and GBS.

## 2. Materials and methods

### 2.1. Patient and control sera

Sera from 129 patients with MG were provided by one of the authors (GTP, UK), all of them with ocular symptoms as the dominant ones. Patients were referred through an eye casualty department to the neuro-ophthalmology clinic and followed by GTP. MG patients were included using the following inclusion criteria: recent onset (within last 2 years) of fatiguable diplopia, ptosis due to levator dysfunction, or both in the absence of generalized weakness. Clinically supportive criteria were the presence of Cogan's lid twitch sign and normal velocity initiation of saccadic eye movements. Supportive electrodiagnostic criteria were the presence of >10% decrement of the compound action potential using repetitive stimulation at 2–3 Hz, and increased jitter using single fibre electromyography. Supportive laboratory tests were the presence of anti-AChR, tested in all patients, or anti-MuSK. An intravenous edrophonium test was performed in a selected group of anti-AChR and anti-MuSK negative patients. Seronegative patients also underwent magnetic resonance brain imaging. All patients were screened for the presence of a thymoma. Sera from 41 blood donors were used as healthy controls. Sera from 49 MS patients (Norway) (87% with relapsing remitting and 13% with primary progressive MS) and 37 GBS patients (Norway) were used as disease controls. Mean MS disease duration was 6.1 years, with a standard deviation of 3.9 years. None of the MS and GBS patients had received any immunoactive treatment (including plasmapheresis) before serum sampling. Samples from GBS patients were collected within the first two weeks of disease onset. Some MG patients were using immunosuppressants and/or acetylcholine esterase inhibitor. All serum samples were stored as 1–2 ml aliquots below  $-20^{\circ}\text{C}$ .

The blood donors were assumed healthy, since the regulations in Norway do not allow people with any acute or chronic diseases to donate blood. The samples were randomly selected from all available blood donors, 77% aged 26–55 years.

### 2.2. Experimental setup

Anti-Hsp70 antibody total (IgG, IgM and IgA) concentrations were measured using an anti-Human Hsp70 ELISA kit (Stressgen, MI, USA) according to assay instructions. All serum samples were tested at a 1:1000 dilution as recommended by the supplier. All experiments were performed in duplicates. The optical density was measured at 450 nm with 590 nm correction using Thermo Multiskan EX plate reader. According to the information given by the manufacturer, the sensitivity of the assay was 6.79 ng/ml. Both the inter- and intra-assay

coefficients of variation were <10%. All readings were within the standard curve.

### 2.3. Statistics

Statistical analysis was performed using Minitab 14. Normality was assessed with the Anderson–Darling test. For continuous variables, group comparisons were assessed using the 2-tailed Mann–Whitney *U* test or 2-tailed *t*-test, depending on normality assessment outcome. Chi-square tests were applied when comparing categorical variables. A *p*-value below 0.05 was considered significant.

## 3. Results

The 129 MG patients consisted of 66 males and 63 females. Their mean age at time of inclusion ( $\pm$ SD) was 57.1 years ( $\pm$ 17.7). 94 (73%) patients had generalized MG (GMG), whereas 35 (27%) had purely ocular phenotype (OMG). Patients classified as OMG had neither electrophysiological nor clinical evidence of limb involvement. Of the 94 GMG patients 36 had detectable levels of anti-AChR antibodies, 53 were negative and 5 unknown. The OMG group had 16 anti-AChR positive, 18 negative and 1 unknown. The anti-Hsp70 concentrations did not fit the normal distribution curve for any of the groups (Table 1).

The GMG and OMG groups did not differ in age ( $p=0.37$ ), sex ( $p=0.42$ ) or anti-AChR antibody status ( $p=0.54$ ). No significant difference in AChR antibody concentration was found when comparing early onset (33%) and late onset (66%) patients in the entire MG group ( $p=0.78$ ), or when comparing GMG and OMG AChR-status and sex sub-groups. Anti-Hsp70 levels did not differ between anti-AChR positive and -negative MG patients (Table 2).

Both GMG and OMG patients had higher anti-Hsp70 concentrations compared to healthy controls ( $p<0.001$ ); median values being 271.0, 333.0 and 171.9  $\mu\text{g/ml}$ , respectively, and compared to MS patients ( $p=0.0004$ ); median value for MS 209.1  $\mu\text{g/ml}$ . Anti-Hsp70 concentrations did not differ between MS patients and healthy controls ( $p=0.45$ ). GBS patients had a median anti-Hsp70 concentration of 471.8  $\mu\text{g/ml}$ , significantly higher than both healthy controls ( $p<0.001$ ), MG patients ( $p<0.0044$ ) and MS patients ( $p<0.001$ ) (Fig. 1).

There was no correlation between patients' age at time of inclusion and anti-Hsp70 concentration in the MG population (data not shown).

## 4. Discussion

We have shown that patients with MG and ocular muscle weakness have increased circulating levels of anti-Hsp70 antibodies compared to healthy blood donors and patients with MS. Higher levels of anti-Hsp70 antibodies are likely to reflect increased exposure to Hsp70 antigens, current or previous, in the circulation or bound to cell membranes. Our results therefore indicate increased exposure to Hsp70 in the course of MG development.

OMG affects a few small muscles only, and patients experience mild symptoms compared to generalized MG. It is interesting that the

**Table 1**  
Characterization of MG patients. (\*Chi-square tests, †*t*-test).

	<i>n</i>	AChR-Ab	Age at inclusion (mean $\pm$ SD)	Sex (m:f)
GMG	94	36 Positive	56.2 $\pm$ 16.8	50 Male
		53 Negative		44 Female
		5 Unknown		
OMG	35	16 Positive	59.5 $\pm$ 17.9	16 Male
		18 Negative		19 Female
		1 Unknown		
<i>p</i> -value		0.54*	0.37†	0.42*

**Table 2**

Anti-Hsp70 antibody concentrations in subgroups of MG patients expressed as median values with interquartile range (IQR) in parenthesis. No significant differences were detected between AChR-positive and -negative patients in both OMG and GMG groups or in the MG group as a whole.

	Median anti-Hsp70 concentrations ( $\mu\text{g/ml}$ )			
	Anti-AChR		Sex	
	Positive	Negative	Male	Female
GMG	299.5	258.3	258.7	286.8
IQR	(287.5)	(352.6)	(323.2)	(261.4)
OMG	387.8	304.1	331.6	333.0
IQR	(873.0)	(317.9)	(357.8)	(508.0)

patients still have significantly increased anti-Hsp70 levels in serum, even without anti-AChR antibodies. This supports OMG as a more generalized autoimmune disease. The same anti-Hsp70 increase in OMG with or without anti-AChR antibodies indicates similar pathogenic mechanisms for these two disease entities, irrespective of anti-muscle antibody status. No difference in anti-Hsp70 concentration was found between OMG and GMG patients, supporting the theory that OMG is a systemic disease similar to GMG.

47% of our OMG patients had detectable levels of anti-AChR antibodies, as expected for OMG (Luchanok and Kaminski, 2008). Surprisingly, only 38.3% of patients with GMG were anti-AChR positive, in contrast to the expected 80–90% (Conti-Fine et al., 2006). This can be explained by the recruitment exclusively of patients presenting to a neuro-ophthalmology clinic with primarily periocular symptoms. Where there was evidence of generalized disease the symptoms and signs tended to be mild. We could find no

difference between anti-AChR positive and negative OMG and GMG patients. Over half of the MG patients considered sero-negative after testing for antibodies using conventional assays have low-affinity antibodies binding to AChR in a cell culture based method (Leite et al., 2008). It is therefore reasonable to assume that a great portion of our anti-AChR negative MG patients have low-affinity anti-AChR antibodies.

Interestingly, we found that GBS patients had increased levels of anti-Hsp70 antibodies compared to all other groups. Increased anti-Hsp70 levels have been reported in cerebrospinal fluid from patients with acute and untreated GBS when compared to patients with motor neuron disease, but no difference was found when comparing concentrations in GBS sera to healthy controls (Yonekura et al., 2004). The diverging results in our and the previous mentioned study may be due to methodological differences as we measured concentrations of total (IgG, IgA and IgM) immunoglobulin, whereas they measured IgG levels only. In an animal model of GBS, upregulation in IL-12 mRNA together with sciatic nerve infiltration of cells expressing Hsp70 was found. These findings correlated with disease severity and sciatic nerve infiltration of Toll-like receptor 2 positive cells (Zhang et al., 2009). Hsp70 is a ligand of the Toll-like receptor 2 with downstream activation of the IL-12 promotor (Vabulas et al., 2002). IL-12 is involved in regulating the Th1/Th2-balance, with increased expression favoring the Th1-response (Gately et al., 1998). Alteration of Hsp70-signaling by antibody-binding may skew the immune response towards Th2, yielding a different disease pathogenesis and phenotype.

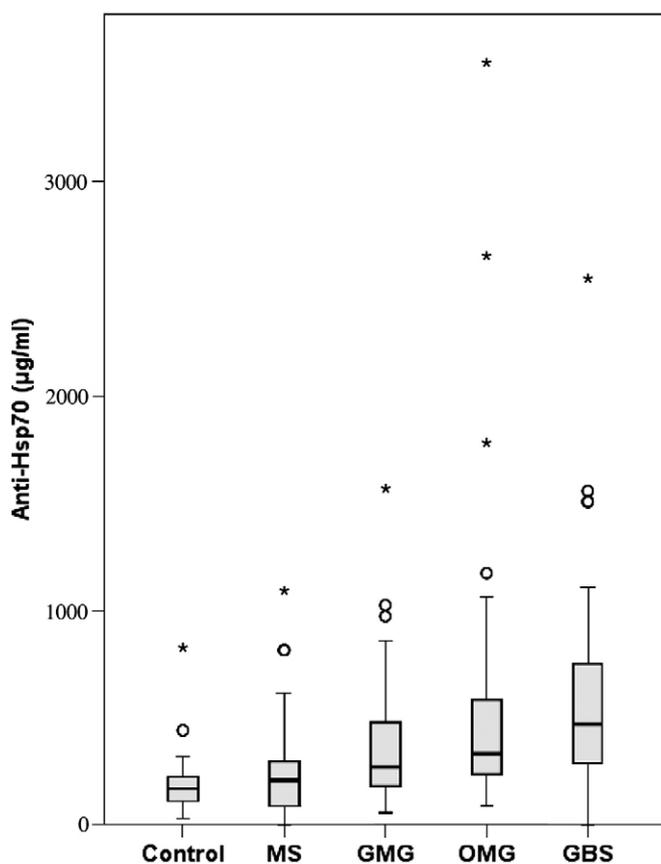
We were unable to examine the correlation between anti-Hsp70 levels and disease severity. All samples from the GBS-patients were obtained within a couple of weeks of disease onset, before any treatment and during active and progressive disease. The MG patients had a relatively mild disease phenotype with symptoms predominantly affecting ocular and periocular muscles. The observation that anti-Hsp70 levels are increased in both GBS and MG, but not in MS, indicates that antibodies against Hsp70 are not disease specific. However, they may reflect ongoing autoimmune disease activity in the peripheral nervous system, and a previous or current increased exposure to Hsp70 antigens.

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**Fig. 1.** Box plot illustrating the anti-Hsp70 antibody concentrations in OMG patients and controls. The boxes represent interquartile range (IQR). Outliers within 1.5 IQR of the boxes are marked with whiskers, whereas outliers between 1.5 and 3 IQR, and 3 IQR of the boxes are denoted ○ and \*, respectively.

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