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# Epidemiological and immunological profile of muscle-specific kinase myasthenia gravis in Greece

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**Objectives:** The purposes of this study were to determine the epidemiological characteristics of muscle-specific kinase-myasthenia gravis (MuSK-MG) in Greece and the IgG subclass of the anti-MuSK antibodies.

**Methods:** This population-based study was performed on MuSK-MG patients in Greece between 1 January 1986 and 30 June 2006. Epidemiological and clinical data for 33 patients were collected. In addition, the distribution of anti-MuSK IgG autoantibody subclasses in the sera of 14 patients was determined by immunoprecipitation.

**Results:** The average annual incidence was 0.32 patients/million population/year. On 1st July 2006, there were 33 prevalent cases, giving a point prevalence rate of 2.92/million (women 4.56 and men 1.25). In females, onset of MuSK-MG occurred after the age of 30, whilst, in males, the disease appears in any decade. The female:male incidence ratio was 3.33:1, whilst the prevalence ratio was 3.65:1. Most patients presented with involvement of the facial and bulbar muscles. Amongst about 800 MG patients seropositive for antibodies against either the AChR or MuSK, one patient was found to be seropositive for anti-MuSK antibodies and ambiguous for anti-acetylcholine receptor (anti-AChR) antibodies. The vast majority of anti-MuSK antibodies were IgG4, whilst total IgG4 levels in these patients were similar to those in two healthy controls.

**Conclusions:** The incidence and prevalence of MuSK-MG in Greece are amongst the highest reported previously for other countries. MuSK-MG in Greece affects both sexes, but mainly females. The main epidemiological indices were calculated. The vast majority of anti-MuSK antibodies were IgG4.

## Introduction

Myasthenia gravis (MG) is an autoimmune disease which affects the neuromuscular junction of the skeletal muscles. In about 85% of patients with generalized MG, pathogenic autoantibodies against the muscle nicotinic acetylcholine receptor (AChR) lead to receptor loss, impaired neuromuscular signal transmission, and muscular weakness and fatigability [1]. The presence of these circulating autoantibodies confirms the diagnosis of the disease. However, in about 15% of patients with generalized MG, no such antibodies are detected using the established anti-AChR radioimmunoassay (RIA) [2]. Interestingly, antibodies against muscle-specific kinase (MuSK) are detected in a number of these patients [3,4]. The proportion of anti-AChR antibody-negative MG patients who have anti-MuSK antibodies seems to vary considerably in different regions (e.g. 47% in the North American population versus 4% in Taiwan and a total absence in Norway) [5–8].

Myasthenia gravis patients with anti-MuSK antibodies constitute a specific subgroup of MG patients. These antibodies may play a role in the impairment of neuromuscular transmission, because MuSK is involved in post-synaptic differentiation and the clustering of AChRs [9]. MuSK is a transmembrane protein which is selectively expressed in skeletal muscles. It consists of four extracellular immunoglobulin (Ig)-like domains, one extracellular Cys-rich domain, and an intracellular kinase domain. MuSK seems to be part of a receptor for agrin, a protein secreted by nerves, which is involved in the formation of the neuromuscular

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junction [10]. MuSK activation by agrin as a result of tyrosine phosphorylation leads to the phosphorylation of rapsyn (a cytoskeletal protein), which, in turn, results in the clustering of AChRs and the phosphorylation of the AChR $\beta$-subunit [9,11].

Despite the absence of anti-AChR antibodies, MuSK-MG is often characterized by moderate to severe MG symptoms. Patients with MuSK-MG present predominantly with bulbar and ocular symptoms, dysphagia and dysarthria, and facial muscle symptoms and show less thymic pathology, more frequent respiratory crises, and a less satisfactory response to immunosuppressive treatment than MG patients with anti-AChR antibodies [12].

Experimental induction of MuSK-MG in animals has been reported in two publications. Immunization of rabbits with the extracellular domain of mouse MuSK led to the development of muscular weakness and this clinical sign, characteristic of MG, was confirmed electromyographically [13]. In another study, injection of the recombinant extracellular domain of rat MuSK into mice resulted in fatigability, tremors, weight loss and death in some strains [14]. This suggests that the immunization of rodents with the extracellular domain of MuSK produces myasthenic symptoms. However, further research is needed to shed light on the mechanisms that cause experimental MuSK-MG.

The epidemiology of MuSK-MG in Greece has not been reported. In this work, we present the clinical and epidemiological data from a national study on a series of 33 MuSK-MG patients. This is a relatively large population study and provides useful data on the natural history of the disease. In addition, we determined the distribution of anti-MuSK antibodies amongst the IgG subclasses in a number of MuSK-MG sera and showed that most were IgG4, in agreement with the results of previous studies [4,15]. Since it is not known how anti-MuSK antibodies result in neuromuscular defects, these findings may contribute to the elucidation of the specific role of these autoantibodies.

## Methods

### Collection of the epidemiological data

We performed a population-based study on MuSK-MG patients in Greece between 1 January 1986 and 30 June 2006. Information on the size of the population of Greece was obtained from the National Office of Statistics. Based on the 2001 national census, the population in 2006 was estimated to be 11 293 282 (5 591 257 males and 5 702 025 females). Forty-one MuSK-MG patients with anti-MuSK antibodies were identified but 33 were finally included in the study, because eight could not be found or denied to reply to the relevant questionnaire. The Hellenic Pasteur Institute is the main institution in Greece in which serum samples are tested for the presence of anti-MuSK autoantibodies. The Greek Personal Data Protection Authority issued a license to the authors to establish and operate at the Hellenic Pasteur Institute a file with sensitive data relevant to diagnosis and therapy of muscular diseases (license no 918). In addition, the Ethics Subcommittee of the Board of Directors of the Hellenic Pasteur Institute issued permission for the present study. Patients gave their informed consent for the study.

A detailed questionnaire was produced and included the patient's name, sex, date of birth, presence of other (especially autoimmune) diseases, relatives with MuSK-MG, clinical manifestations, course and progression of the disease, therapies used, any relevant epidemiological information, and the anti-MuSK antibody titer, and the patients were invited to participate in the study. The basic data in this study were obtained by personal interview, telephone or letter. Incidence was based on the year of clinical onset. MuSK-MG patients were considered prevalent if they were living in Greece on 1 July 2006 (prevalence day).

### Assay of total anti-MuSK antibody titer.

The measurement of anti-MuSK antibodies was performed using an anti-MuSK Ab assay kit (RSR Ltd, Cardiff, UK) according to the manufacturer's instructions with slight modifications. Briefly, the diluted sera were incubated overnight at 4°C with 25 $\mu$l (about 30 000 cpm) of $^{125}$I-MuSK solution, then the $^{125}$I-MuSK-antibody complexes were precipitated by incubation for 2 h at 4°C with 25 $\mu$l of anti-human IgG antibody and centrifugation, and the precipitated radioactivity measured in a $\gamma$-counter (2470 WIZARD$^{2™}$, PerkinElmer 2470 WIZARD$^{2TM}$, Turku, Finland).

### Anti-MuSK IgG antibody subclass determination

The IgG subclass of the anti-MuSK Abs was tested by immunoprecipitation using a MuSK Ab Assay kit, as described previously [4], with slight modifications. In brief, 25 $\mu$l of $^{125}$I-MuSK solution was incubated with 0.3 $\mu$l of the patient's serum for 4 h (at 4°C), then 5 $\mu$l of 1 mg/ml of affinity-purified sheep antibodies against human IgG1, IgG2, IgG3 or IgG4 (Binding Site, Birmingham, UK) was added and the mixture left overnight at 4°C for the formation of immune complexes. Normal human serum was used as negative control. The immune complexes were then precipitated with anti-sheep IgG antiserum, previously depleted of antibodies cross-reactive with human Ig. The precipitated $^{125}$I-MuSK was washed and counted in a $\gamma$-counter.

### Immobilization of anti-IgG4 antibodies on CNBr–Sepharose beads and immunoadsorption of MuSK-MG autoantibodies

The affinity-purified anti-human IgG4 antibodies, which were found to bind to the vast majority of the anti-MuSK IgG antibodies, were immobilized on Cyanogen Bromide-activated Sepharose 4B (CNBr-activated Sepharose 4B) beads (Pharmacia-Biotech, Amersham Biosciences AB, Uppsala, Sweden), according to the manufacturer's protocol. In brief, 0.8 mg of anti-human IgG4 antibody in 1 ml of coupling buffer (0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl) was incubated with 0.28 g of CNBr-activated Sepharose for 2 h at room temperature, then overnight at 4°C. Any remaining active groups on the Sepharose were blocked by incubation for 2 h at room temperature in 0.1 M Tris–HCl, pH 8.0 and the beads washed with three cycles of 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl, followed by 0.1 M Tris–HCl, pH 8.0, containing 0.5 M NaCl. The Sepharose-anti-IgG4 antibody conjugate was stored in phosphate-buffed saline, pH 7.4, containing 0.05% sodium azide. Immunoadsorption experiments were performed by incubating 3 $\mu$l of anti-MuSK-MG antibody-containing serum with 27 $\mu$g of anti-human IgG4 antibody immobilized on Sepharose beads for 12 h at 4°C with gentle agitation, then assaying 0.3 $\mu$l of the treated serum using the anti-subclass sera as described above.

## Results

### Epidemiology

Thirty-three MG patients were identified as anti-MuSK antibody-positive. Female patients displayed a strikingly high prevalence compared to male patients, with 26 patients being women (78.8%) and only seven men (21.2%) (female:male ratio 3.70:1). Age at disease onset ranged from 6 to 74 years. In females, onset of MuSK-MG occurred after the age of 30, whilst, in males, it occurred in all decades (Fig. 1). No familial cases were identified.

### Incidence and prevalence

The average annual incidence rate was 0.32 patients/million population/year. The annual female incidence rate was 0.50 patients/million population/year, whilst that for males was 0.148. Consequently, the female:male annual incidence ratio was 3.33:1 (Fig. 2). The day chosen for prevalence determination was 1 July 2006, on which date 33 patients (26 women and seven men) diagnosed with MuSK-MG were living in Greece. The point prevalence rate on this day was 2.92/million population. By sex, the prevalence was 4.55/million females and 1.25/million males, a female:male prevalence ratio of 3.65:1.

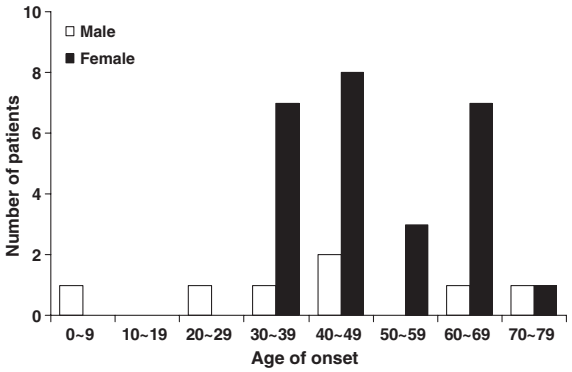


**Figure 1** Age at which symptoms first appeared in the muscle-specific kinase (MuSK)-positive patients.

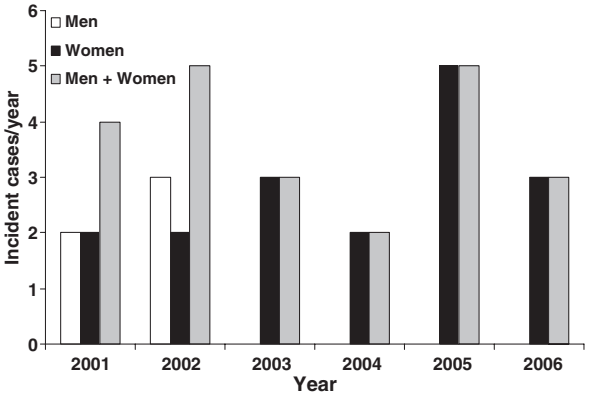


**Figure 2** Annual new cases of muscle-specific kinase-myasthenia gravis (MuSK-MG).

### Clinical features

Muscle-specific kinase-myasthenia gravis MuSK-MG is characterized by a stable course and is less responsive to conventional treatments [12]. The patients in this study showed acute or subacute onset of the disease. In the majority of cases, the presenting symptoms were diplopia, difficulty in swallowing, drooping of the eyelids and nasal speech. Seven patients presented with limb muscle weakness during the initial assessment. Six patients presented with respiratory crises because of the involvement of the respiratory muscles. They were treated in an Intensive Care Unit and endotracheal intubation with artificial ventilation was applied, whilst the other 27 presented with ophthalmoparesis. In 19 patients, the first symptoms of the disease were bulbar (Table 1). None of the 33 patients had a thymoma or

**Table 1.** Prevailing MG symptoms at disease onset

| | Males (%) | Females (%) |
|---|---|---|
| MuSK-MG patients | 7 | 26 |
| Limb weakness | 3 (45) | 4 (16) |
| Respiratory crises | 3 (45) | 3 (12) |
| Ophthalmoparesis | 5 (70) | 22 (80) |
| Bulbar weakness | 4 (58) | 15 (58) |

MuSK-MG, muscle-specific kinase-myasthenia gravis.
The most frequent clinical symptoms at the time of presentation are shown.

had undergone thymectomy. Associated disorders were reported by eight of the patients, these being diabetes mellitus (one patient), thyroid abnormality (five patients), Hashimoto's disease (one patient) and Crohn's disease (one patient).

### MuSK antibody subclass determination

Anti-MuSK antibody-positive serum samples from 14 patients randomly selected were tested for anti-MuSK antibody subclass using affinity-purified IgG subclass-specific antibodies in the immunoprecipitation assay. All showed a predominance of IgG4 (Fig. 3). Small amounts of $^{125}$I-MuSK were also precipitated by antibodies to other subclasses. In order to verify whether the values for the IgG1, IgG2 and IgG3 subclasses were specific or were due to cross-reaction of the anti-subtype antibodies with other subtypes, six of these MuSK-MG sera (Nos 1–6 in Fig. 3) were randomly selected and pre-incubated with immobilized anti-human IgG4 antibodies, then the IgG4-depleted sera were subjected to RIA for subclass determination. As shown in Fig. 4, in addition to the IgG4 antibodies, antibodies of the other three subclasses were significantly adsorbed by the anti-IgG4 immunoadsorbent,

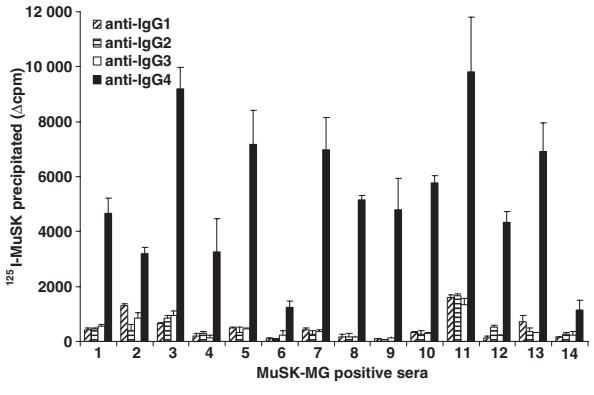


**Figure 3** IgG subclass determination of the anti-MuSK antibodies in 14 sera by immunoprecipitation. The values are the mean of three independent experiments ± SEM.

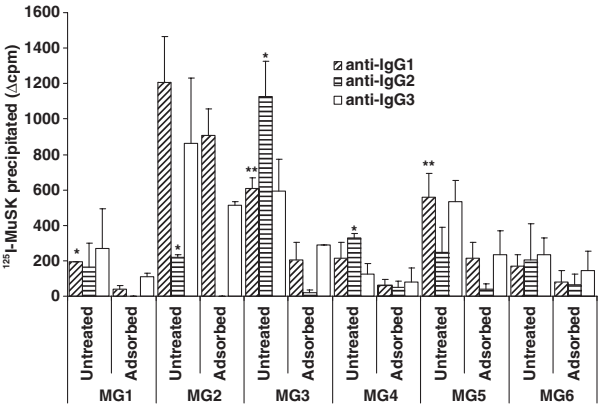


**Figure 4** Effect of IgG4 antibody depletion on the apparent IgG subclass specificity of anti-MuSK antibodies. Six MuSK-MG sera were incubated with anti-human IgG4 antibody bound to Sepharose beads, then the treated and untreated sera were tested by radioimmunoassay using the four anti-IgG subclass-specific antibodies. In addition to the expected dramatic decrease in the antibodies precipitated by anti-IgG4 antibodies, a large fraction of the antibodies initially shown to be precipitated by the anti-IgG1, anti-IgG2, or anti-IgG3 antibodies were also adsorbed by the immobilized anti-IgG4 antibodies. The results are expressed as the mean of three independent experiments ± SEM (*$P < 0.05$; **$P < 0.1$ in Student's *t*-test).

the difference often being statistically significant ($P < 0.05$ or $P < 0.1$, Student's *t*-test). This suggests that the anti-subclass antisera are not strictly subclass-specific and that many, if not all, of the anti-MuSK antibodies precipitated by the affinity-purified antibodies against IgG1, IgG2 or IgG3 (Fig. 3) were, in fact, IgG4.

Taking into account that the vast majority of the anti-MuSK antibodies were IgG4, we assayed the 14 MuSK-MG sera by ELISA for total IgG4 and found levels were similar to those in two normal human sera (data not shown).

In general, anti-MuSK antibodies do not coincide with anti-AChR antibodies. We examined 813 anti-AChR (780) or anti-MuSK (33) antibody-positive-MG sera for the presence of the other antibody. Interestingly, one patient who was positive for anti-MuSK antibodies (titer 10 nM) was found to have an ambiguous anti-AChR antibody titer (0.3 nM). This patient was not included in the above study, because the disease appeared after the prevalence date. All other sera were positive for only one of the two antibodies.

## Discussion

The identification of anti-MuSK antibodies in patients' sera constitutes considerable progress in the understanding and diagnosis of non-anti-AChR MG. The present study is the first report of a MuSK-MG survey

in Greece. Previous reports have indicated a different prevalence of MuSK-MG in different countries (reviewed in [16]). It is noteworthy that the prevalence of MuSK-MG is low in Taiwan, high in North America, and currently zero in Norway [7]. This shows that environmental and genetic factors may play a role in the initiation of the disease [8,17]. According to this general observation, Greece was expected to have a relatively high incidence of MuSK-MG and this was confirmed by our findings.

The average annual incidence in our study was 0.32 patients/million population/year, whilst the point prevalence rate on the day chosen was 2.92 patients/million population. A clear female preponderance was a significant feature of our anti-MuSK antibody-positive patients, which is in accordance with the findings of other studies [12,16,18]. No familial cases of MuSK-MG were seen. We found a predominant involvement of the ocular and bulbar muscles, as in other studies (reviewed in [12]).

The experimental aim of our study was to determine the IgG subclass of the anti-MuSK antibodies. We tested 14 sera and our findings confirmed those of previous studies showing a predominance of IgG4 amongst the anti-MuSK antibodies [4,15]. Furthermore, we showed that even the small fraction of antibodies precipitated by antibodies against IgG1, IgG2 or IgG3 may actually be IgG4, as a result of cross-reaction with the anti-subclass antibodies used.

Increased total IgG4 levels have been observed in patients with the autoimmune disease sclerosing pancreatitis, the average total serum IgG4 concentration in these patients being 663 mg/dl, compared with 51 mg/dl in normal human serum [19]. In order to test whether the IgG4 predominance amongst the anti-MuSK antibodies was due to a higher level of total IgG4 in the MG patients in our study, we compared total IgG4 levels in the 14 MG sera and in two healthy controls and found they were similar.

If MuSK-MG is due to the IgG4 antibodies, the pathogenic mechanism of these anti-MuSK antibodies should be quite different to that of anti-AChR antibodies. Anti-AChR antibodies seem to act mainly by causing AChR loss through the binding and activation of complement and induction of AChR antigenic modulation (induced by antibody-mediated cross linking). In contrast, IgG4 antibodies do not bind complement and seem to be functionally monovalent [20]. Thus, the anti-MuSK antibodies seem to exert their effect by directly blocking MuSK function. Further studies are required to elucidate the exact role of anti-MuSK antibodies in the initiation and pathogenesis of MuSK-MG.

## Acknowledgements

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