# Myasthenia gravis with antibodies to muscle-specific kinase : clinical characteristics, epidemiology, and immunological aspects

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# Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1

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*Journal of Neuroimmunology 2008;195:151-156*

## Abstract

We studied the longitudinal relation between disease severity and titres of antigen-specific IgG subclasses in sera of patients with myasthenia gravis and antibodies to muscle-specific kinase (MuSK MG). Six patients were included of whom 55 samples had been collected during 2.5-13.4 years. Anti-MuSK antibodies were determined by ELISA and with a cell-based immunofluorescence assay. Disease severity was scored on a semi-continuous scale. Only antigen-specific IgG4, and not IgG1, titres were significantly associated with disease severity in a linear mixed effect model ($p = 0.036$). Levels of IgG4 antibodies were above IgG1 in all samples except in one patient who went into clinical remission while switching from IgG4 to IgG1. The results support an important role for IgG4 in the pathogenesis of MuSK MG, in contrast to MG with anti-acetylcholine receptor antibodies.

## Introduction

Myasthenia gravis with autoantibodies against muscle-specific kinase (MuSK MG) is a rare disorder of neuromuscular transmission predominantly affecting craniobulbar and respiratory muscles. [56] In mice, MuSK is involved in the organisation and maintenance of the neuromuscular junction through clustering of postsynaptic acetylcholine receptors (AChR). [65] Immunization of mice with the extracellular domain of the MuSK receptor induces a postsynaptic neuromuscular transmission disorder with exercise-induced weakness. [124] In mammalian muscle, RNA interference with MuSK induces the disassembly of existing neuromuscular junctions underscoring the role of MuSK in synapse maintenance. [66] The deficiency of synaptic transmission can be confirmed by EMG showing a decrement of the compound muscle action potential upon repetitive nerve stimulation or an increased jitter in single-fiber EMG. In MuSK MG autoantibodies are predominantly of the IgG4 subclass, although IgG1 autoantibodies are also present. [93] This is in contrast to myasthenia gravis with anti-acetylcholine receptor antibodies (AChR MG), where IgG1 and IgG3 autoantibodies cause postsynaptic complement deposition, AChR depletion and structural damage to the neuromuscular synapse. Such histological abnormalities have not been found in patients with MuSK MG [125] and it has been suggested that anti-MuSK antibodies may not be the primary cause of myasthenic symptoms. [126] In order to define better the pathogenic role of these antibodies, we investigated the longitudinal association between titres of anti-MuSK specific IgG subclasses and disease severity in MuSK MG.

## Patients and methods

### Patient selection

All consecutive patients with MuSK MG, registered prospectively in our centre since 1990, were included who had been followed clinically for more than three years and from whom multiple serum samples were available. Samples had been collected for future research throughout the period of clinical follow-up. Presence of anti-MuSK antibodies and absence of anti-AChR antibodies were confirmed using standard commercial assays (RSR Ltd., Pentwyn, Cardiff, UK).

### Disease Severity Score for grading clinical symptoms

Severity of MG symptoms was evaluated retrospectively and independently by two neurologists with extensive clinical experience in neuromuscular disorders (PWW and ARW). Both neurologists were blinded for the anti-MuSK antibody titres. They were informed on what date each serum sample had been obtained and used information from

patients' charts to evaluate the severity of symptoms on that date. The Disease Severity Score (DSS) was a semi-continuous variable between 1 and 5, allowing regression analysis. The definitions of the units in this scale, shown in Table 5.1, were modified after Oosterhuis *et al*. [37] in combination with the grading system of bulbar symptoms according to Brooke. [127] Tenth-values were used to allow scoring of subtle fluctuations of disease severity. The results of the DSS which were used for the statistical analyses were based on consensus between the two neurologists. Symptoms on each time point were also classified according to the MGFA clinical classification. [113] This scale was not used for statistical analyses because of its ordinal feature.

**Table 5.1 Definition of units for the Disease Severity Score (DSS)**

| | |
|---|---|
| 1 | Complete remission, no signs after exertion, no complaints. Speech and swallowing are normal |
| 2 | Minor signs or minor complaints. No disability. The experienced doctor may find minor signs at appropriate testing. Speech or swallowing may be abnormal but do not cause practical problems |
| 3 | Moderate disability. Clear signs after exertion. Some restrictions in daily life. Speech is difficult to understand. Swallowing may cause choking |
| 4 | Severe disability, clear signs at rest. Restrictions in domestic activities. Speech is intelligible for relatives or close friends but unintelligible for others. Difficulties with swallowing are constant and meals have to be adapted. Decreased respiratory function may be present |
| 5 | Respiratory support |

**ELISA for anti-MuSK specific IgG antibodies**

Maxisorp plates (96 well, NUNC, Roskilde, Denmark) were coated overnight at 4 °C with 100 µl/well of 0.4 µg/ml of the extracellular domain of human MuSK [93] dissolved in phosphate buffered saline pH 7.2 (PBS) and blocked for 1 hour at room temperature (RT) with 150 µl/well 2% Casein in PBS with 0.05 % Tween (PBS/C/T).

The plates were incubated with 3-fold serial dilutions of serum samples and standards, starting with a 1:50 dilution, in PBS/C/T for 2 hours at RT. For the detection of total IgG anti-MuSK, the plates were incubated for 2 hours at RT with alkaline phosphatase-conjugated goat-anti-human IgG (1:2000, Invitrogen, Carlsbad, California, USA). Anti-MuSK antibodies in the different IgG subclasses were measured by a two hours incubation at RT with specific monoclonal antibodies (IgG1, MH 161-1, Sanquin, Amsterdam, The Netherlands; IgG2, HP6014; IgG3 NI86; IgG4 NI315, Nordic, Tilburg, The Netherlands), followed by incubation overnight at 4 °C with alkaline phosphatase-conjugated rabbit anti-mouse Ig (1:750, Dakopatts, Glostrup, Denmark). After incubation with substrate (*p*-nitrophenylphosphate) in 0.01 M diethanolamine buffer pH 9.8 containing 1 mM $MgCl_2$, the reaction was stopped with 3 M NaOH. The optical density (OD) at 405 nm was recorded with a Versamax microplate reader. Standard curves and calculations were made using the Softmax pro software (Molecular Devices, Sunnyvale, California, USA). The concentration of

anti-MuSK antibodies, expressed as arbitrary units (AU) per ml, was calculated by comparison with calibration lines from serial dilutions of a serum sample from a severely affected MuSK MG patient, not included in the present study. This serum contained relatively high titres of anti-MuSK specific IgG1 and IgG4 antibodies and was used as an internal standard.

**Comparison of ELISA titres of anti-MuSK specific IgG subclasses**

OD values obtained by using four different monoclonal mouse antibodies, each specific for one of the four human IgG subclasses, represent the relative quantities of the bound human IgG subclass as a proportion of that subclass in the internal standard. Because the exact molar concentrations of the anti-MuSK specific IgG subclasses in the standard serum are unknown, anti-MuSK specific IgG1 and IgG4 arbitrary units cannot simply be compared. To enable such a comparison and, thereby, calculation of the true ratios of the anti-MuSK specific IgG subclasses, sera of MuSK MG patients containing nearly only anti-MuSK specific IgG4 with a negligible quantity of IgG1 were selected. We postulated that the anti-MuSK specific IgG4 titres in these sera were equal to the total anti-MuSK specific IgG titres. The average ratio of anti-MuSK specific total IgG versus IgG4 was 0.99 and this conversion factor was used to adjust the anti-MuSK specific IgG4 titres in sera of the included patients.
Similarly, a pool was constructed consisting of sera with the highest anti-MuSK specific IgG1 titres. Because, even in this pool, IgG4 could not be neglected, we postulated the sum of anti-MuSK specific IgG1 and IgG4 to be equal to the total anti-MuSK specific IgG titre. The average ratio of anti-MuSK specific total IgG versus IgG1 after subtraction of the IgG4 titre yielded a factor of 0.09, which was then used to adjust the IgG1 titres of the sera of the included patients. The adjusted titres were used to calculate the anti-MuSK specific IgG4/IgG1 ratios in these sera and to graphically represent anti-MuSK specific IgG1 and IgG4 titres on the same scale.

**Cell-based immunofluorescence assay for anti-MuSK specific IgG antibodies**

HEK 293 cells were grown on coverslips and transiently transfected using polyethylenimine (PEI) with MuSK DNA tagged with enhanced green fluorescent protein (EGFP). Immunofluorescence staining was performed approximately 48 hours after transfection. The coverslips were incubated with patient or control sera (1:20 for total IgG and 1:20, 1:60 and 1:180 for IgG1 or IgG4) for 1 hour at room temperature (RT). Cells were washed, fixed with 3% formaldehyde in PBS for 15 minutes at RT. For the detection of total IgG antibodies, cells were incubated with goat anti-human IgG-Alexa Fluor 568-conjugated antibody (Invitrogen-Molecular Probes, Paisley, UK) at 1:750. For the IgG subclasses, cells were first incubated with mouse anti-human IgG1 or mouse anti-human IgG4 (Binding site, Birmingham, UK) at 1:50, for 1 hour at RT, and then with goat anti-mouse isotype specific IgG-Alexa Fluor 568-conjugated secondary antibody (Invitrogen-Molecular Probes, Paisley, UK) at 1:750,

for 45 minutes at RT. Coverslips were mounted on slides in fluorescence mounting medium (DakoCytomation) with DAPI (4,6-diamidino-2-phenylindole) to counterstain nuclei. They were examined and imaged on a fluorescence microscope (Olympus, London, UK) with a digital camera Hamamatsu and Openlab imaging software (both provided by Improvision, Coventry, UK).

**Calculation of cell-based titres of anti-MuSK specific IgG subclasses**

The complete coverslip was checked for stained cells. They were coded and scored for the frequencies and intensities of surface binding by the human/mouse IgG antibodies. We used the following scoring system systematically for every coverslip: (0) = no labelling; (0.5) = very weak labelling of very few transfected cells with no obvious co-localisation; (1) = weak labelling of some of the transfected cells, with co-localisation; (2) = moderate labelling of some (~ 20-50%) of the transfected cells, with precise co-localisation; (3) = moderate/strong labelling of ~50%-80% of the transfected cells, with perfect co-localisation; (4) = strong labelling of virtually all transfected cells, with perfect co-localisation. The final score was the mean value of two scores by two different experienced people blinded for the condition (control or patient) and for antibody titres. The ratio between IgG4 and IgG1 anti-MuSK antibodies was calculated by dividing the final scores for the optimal dilution.

**Statistical analysis**

A linear mixed effect model was used to asses the associations on group level between anti-MuSK specific IgG1, IgG4 and total IgG titres (on a log scale) and the DSS. This model takes into account multiple measurements per patient and the variability in the number of measurements between patients. *P*-values for this model were calculated with the Wald test. Linear regression analyses were also performed for each patient separately. The interobserver variability of the independent DSS scores was expressed as a coefficient of variation (CV). This CV was calculated as the standard deviation of the two neurologists' scores for each time point divided by the mean of these scores and multiplied by 100 to present as percentage. The level of agreement between the two independent scores for every coverslip in the cell-based assay was expressed by the intraclass correlation coefficient. All calculations were performed for adjusted and non-adjusted ELISA titres using the SPSS 14.0 Statistical software (SPSS Inc, Chicago, Illinois, USA) and SAS 9.1, Proc Mixed (SAS Institute, SAS Institute Inc, Cary, NC, USA).

## Results

Three men (patients A, D and F) and three women (patients B, C and E) were included. Mean age at onset of symptoms was 32.4 years (range 18.7 to 43.2). Fifty-five samples were available (range 7 to 12 per patient) covering a mean period of 4.7 years (range 2.5 to 13.4). The interval between the onset of symptoms and the first available sample varied from 6 months (patient B) to 33 years (patient F). All patients had predominantly oculobulbar symptoms (MGFA class IIb, IIIb, IVb and V) if not in clinical remission.

Anti-MuSK antibodies were mainly IgG4 in all patients except for patient B who made a class switch from IgG4 to IgG1 while going into clinical remission. The mean ratio of adjusted IgG4/IgG1 titres for all patients was 18 (range 2 to 62). None of the patients had detectable anti-MuSK specific IgG2 or IgG3 antibodies. The linear mixed effect model showed a significant association between adjusted IgG4 titres and DSS on a group level (β coefficient 0.44, $p = 0.036$), whereas no association was found for adjusted IgG1 titres (β coefficient -0.32, $p = 0.13$). Total IgG was weakly associated with DSS (Table 5.2). Intraindividually, a positive correlation between anti-MuSK specific IgG4 and the DSS was found in 5 of the 6 patients, but not in patient C. Slopes varied from 0.01 to 0.15 reaching statistical significance in 3 patients ($p < 0.05$, Table 5.2).

**Table 5.2 Disease Severity Score versus adjusted antibody titres**

| | | β coefficient (*p*-value) | | |
|---|---|---|---|---|
| | | IgG4 | IgG1 | IgG total |
| Group level | | 0.44 [a] (0.036)* | -0.32 [a] (0.134) | 0.619 [a] (0.075) |
| Per patient | A | 0.146 (0.064) | -2.576 (0.333) | 0.078 (0.322) |
| | B | 0.060 (<0.001)* | -0.078 (0.238) | 0.068 (0.000)* |
| | C | -0.003 (0.918) | 0.269 (0.641) | -0.001 (0.988) |
| | D | 0.014 (0.004)* | 0.358 (0.015)* | 0.018 (0.054) |
| | E | 0.010 (0.021)* | 0.220 (0.193) | 0.008 (0.015)* |
| | F | 0.013 (0.066) | 0.102 (0.552) | 0.014 (0.074) |

Correlation coefficients representing the relations between the Disease Severity Score and adjusted titres of anti-MuSK specific IgG4, IgG1 and total IgG on group level and individually. [a] β-coefficient on a log scale. * $p < 0.05$.

The apparent correlation in the other two (patient A and F) failed to reach statistical significance. IgG1 was correlated with DSS only in patient D. In this patient relatively high IgG1 titres correlated with a low DSS. Regression slopes and *p* values were similar for adjusted and non-adjusted titres (data not shown). The correlations between DSS and adjusted titres of anti-MuSK specific IgG1 and IgG4 for each patient are shown in Figure 5.1. The interobserver variability of the DSS varied between patients from 3.6% to 26.2% with a median CV of 9.5% (interquartile range 4.3-15.9).

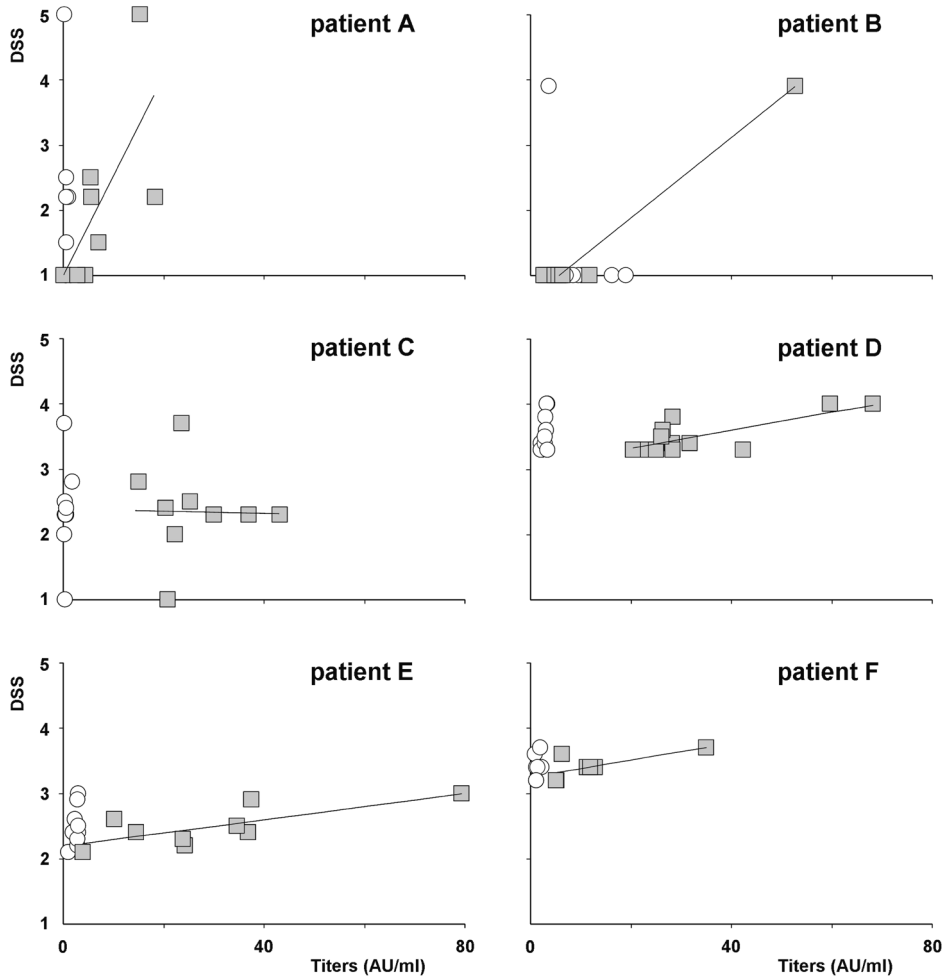


**Figure 5.1 The Disease Severity Score (DSS) versus adjusted titres of anti-MuSK specific IgG1 and IgG4 in arbitrary units (AU) per ml in 6 patients**
○ = IgG-1, □ = IgG-4

Detailed graphical representation of the course of the DSS, adjusted anti-MuSK antibody titres, and the various immunosuppressive treatments per patient are shown in Figure 5.2. All patients had been treated with oral immunosuppressants somewhere during follow-up. Duration of this therapy varied between 38% (patient B) and 100% (patients A, C and D) of the follow-up period. Two patients (C and D) received a single IVIG treatment. Three patients were treated with plasma exchange (A, B and F). Patient A and B were thymectomized within a few months after the onset of symptoms and just before the plasma exchange.

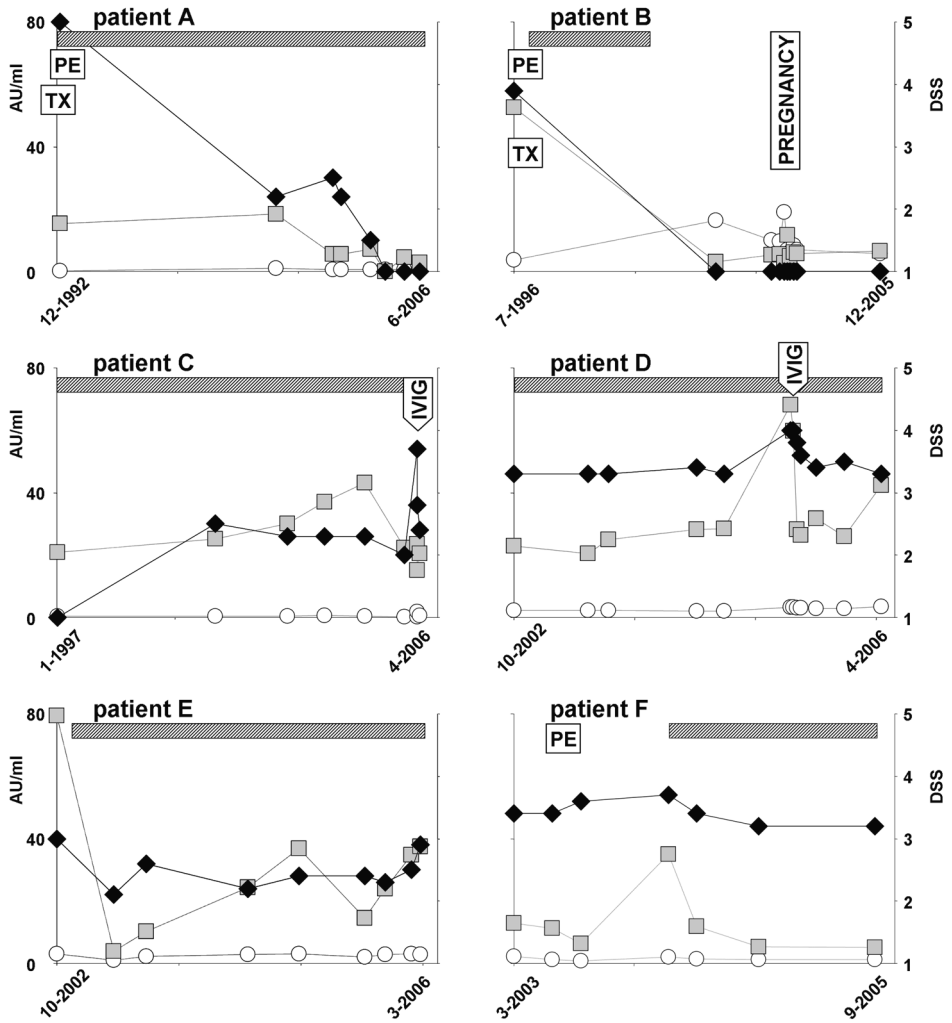


**Figure 5.2 Course of Disease Severity Score (DSS) and adjusted anti-MuSK specific IgG1 and IgG4 titres in AU/ml during follow-up**

○ = IgG-1, □ = IgG-4, ◆ = DSS, ▨ = Oral immunosuppression

PE = Plasma exchange, TX = Thymectomy, IVIG = Intravenous Human Immunoglobulin

The highest titres of IgG1 in our study were detected in patient B. However, when this female patient presented with severe oculobulbar weakness in 1996, anti-MuSK antibodies were predominantly IgG4 (adjusted IgG4/IgG1 ratio 14). After thymectomy and treatment with plasma exchange followed by prednisone for 6 months and azathioprine for 4 years, she was in complete clinical remission in 2001. By then, anti-MuSK antibodies were predominantly IgG1 with an adjusted IgG4/IgG1 ratio of 0.2. During the next 4 years, this ratio fluctuated between 0.1 and 1.7 in nine samples without any signs of clinical relapse.

An example of the use of tenth-values in the DSS, indicating small differences in clinical symptoms, can be observed in patient D. His bulbar symptoms were compatible with DSS category three and too mild for category four, because his speech was still intelligible and meals did not have to be adapted (Table 5.1). After an IVIG treatment in July 2005, he experienced a gradual speech improvement. He could eat his meals quicker than before and reported less shortness of breath at night. Nevertheless, speech and swallowing still caused practical problems and some disability, thereby exceeding category two. The use of tenth-values allowed to score this clinical improvement as a decline in the DSS from 3.8 in July, to 3.6 in August, and 3.4 in September 2005.

To confirm the IgG4 predominance and the changes over time, we used the cell-based immunofluorescence assay, which yielded IgG4/IgG1 ratios of 0.4 to 7, similar to those of the non-adjusted ELISA titres (mean ratio 2, range 0.2 to 6). The intraclass correlation coefficient for the two independent scores was 0.96 ($p < 0.001$). The class switch in patient B was also confirmed by immunofluorescence, yielding IgG4/IgG1 ratios of 3 (1996), 0.4 (2001), and 0.6 (2003). Results are shown in Figure 5.3.

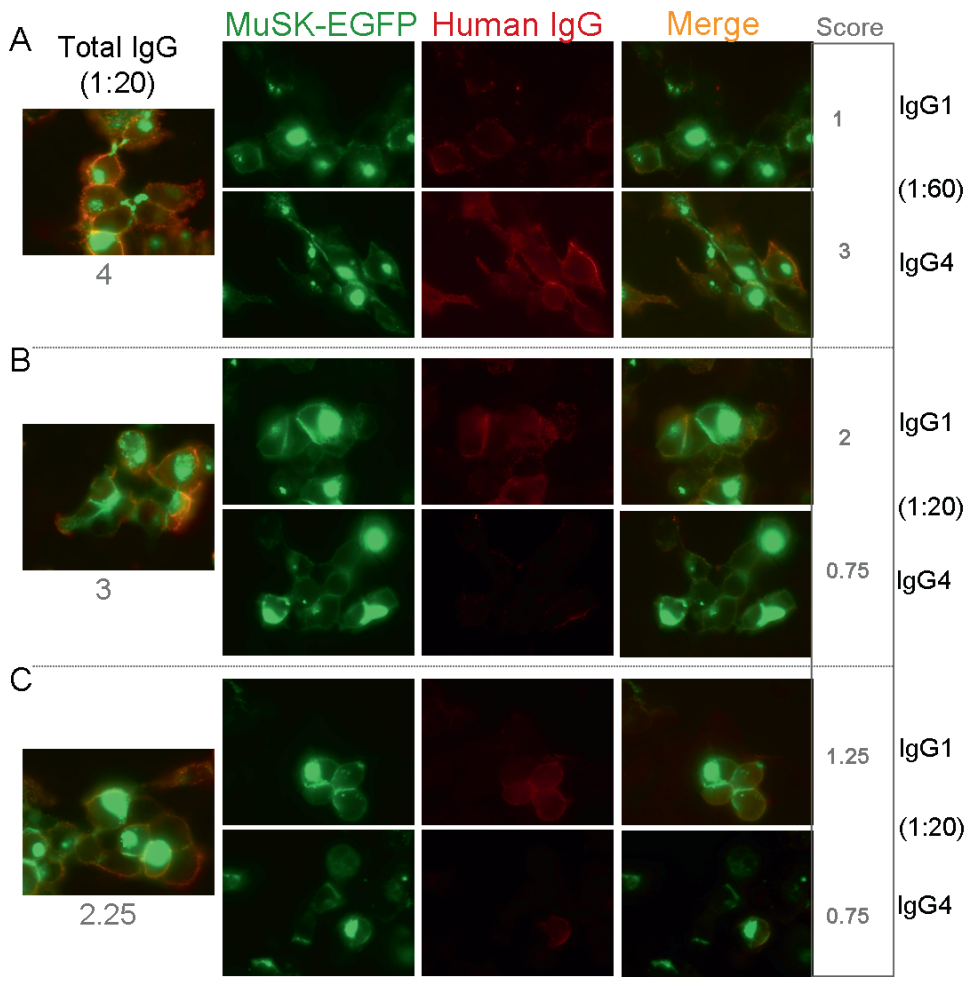


**Figure 5.3 Specific binding of IgG antibodies (red) to MuSK-EGFP on HEK cell surface (green).** A switch from IgG4 to IgG1 anti-MuSK antibodies is shown in three different serum samples from patient B, collected when the patient was severely affected in 1996 (A), and during clinical remission in 2001 (B), and 2003 (C). Stainings are shown for total IgG (1:20), already merged (left), IgG1 (1:60) and IgG4 (1:20) antibodies. Binding scores are also shown (0: negative; 4: highest positive).

## Discussion

In this study, anti-MuSK antibodies were mainly of the IgG4 subclass compared to IgG1. Only anti-MuSK specific IgG4 titres significantly correlated with disease severity on a group level. None of the patients included in this study had IgG2 or IgG3 antibodies against MuSK. IgG2 and IgG3 anti-MuSK antibodies were only detected at low titres in sporadic serum samples of a limited number of MuSK MG patients in the Netherlands (data not shown). Anti-MuSK specific IgG1 correlated with disease severity only in patient D, but the extremely low titres suggest that this correlation is not of clinical significance.

Unfortunately, we could only include 6 patients in this longitudinal study. MuSK MG is a rare disorder which limits the feasibility of large scale prospective research. In spite of the small number of patients we were able to demonstrate an association between disease severity and anti-MuSK specific IgG4 titres. Another possible limitation is the retrospective quantification of the disease severity using a newly developed clinical scale that may have introduced a subjective element. However, existing scales such as the MGFA clinical classification have not been designed to measure outcome and lack quantification. [113] As an ordinal scale, the MGFA clinical classification is not suited for correlation or regression analyses. The quantitative MG score, designed for prospective follow-up, is less suited for patients with predominantly craniobulbar weakness. The DSS provided a possibility to grade smaller fluctuations of symptoms. In general practice, these are often indicated by patients and can be of great importance for clinical decision making. We limited the subjective element by the use of two experienced neurologists who scored the disease severity independently. The considerable level of agreement between these independent scores and the significant association with IgG4 autoantibody titres both imply that the scale was a useful instrument for the retrospective grading of clinical symptoms in patients with MuSK MG.

A correlation between clinical symptoms and total IgG titres of anti-MuSK antibodies has been reported by others. [128] In this study, results of patients included with a single sample and of those included with multiple samples are combined, making it somewhat difficult to discriminate between an interindividual and intraindividual relationship. In AChR MG the interindividual correlation between symptoms and autoantibody titres is weak in contrast to the intraindividual correlation. [37] The predominance of IgG4 anti-MuSK antibodies in MuSK MG has been described previously although up to 30% of anti-MuSK antibodies was IgG1. [93]

An IgG4 immune response is mainly seen after chronic antigen exposure, for example in the humoral response of beekeepers to bee venom. [129] Examples of IgG4 mediated autoimmune diseases are acquired hemophilia A, [130] latent autoimmune diabetes, [131] sclerosing pancreatitis, [132] and pemphigus vulgaris (PV). [123] In PV patients with active disease IgG1 and IgG4 autoantibodies recognise two different epitopes of the PV specific antigen, whereas in patients

with prolonged disease remission, mainly IgG1 autoantibodies against one of these epitopes were found. [123] The authors propose that class switching may be involved in fluctuations of disease severity although no patients with longitudinal follow-up were included. In another study in which sera of 7 pemphigus patients collected during disease exacerbations and remissions were investigated, no evidence for such a class switch was obtained. [133] Therefore, the remarkable switch from IgG4 to IgG1 autoantibodies with a simultaneous clinical remission of MuSK MG in patient B seems a possible but not unique mechanism for disease fluctuations in antibody mediated autoimmune disorders. IgG4 is a functionally monovalent antibody, unable to crosslink or activate complement. Hence, the pathogenic mechanism of IgG4 antibodies to MuSK is more likely to be a direct effect on MuSK receptor function in contrast to the complement mediated destruction of the neuromuscular junction mediated by IgG1 and IgG3 autoantibodies in AChR MG. The results from this study provide an argument for the pathogenic role of IgG4 autoantibodies in MuSK MG although further studies are required to clarify how these autoantibodies interfere with signal transmission in the neuromuscular junction.

## Acknowledgements

The authors thank Dr. Stuart Viegas for help with the staining and analysis of the results of the cell-based immunofluorescence assay. They also thank Dr. Saskia le Cessie for help with statistical analysis.