CHAPTER 4

Longitudinal epitope mapping in MuSK myasthenia gravis: implications for disease severity

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Journal of Neuroimmunology 2016 Feb 15;291:82-8.

ABSTRACT

Muscle weakness in MuSK myasthenia gravis (MG) is caused predominantly by IgG4 antibodies which block MuSK signalling and destabilize neuromuscular junctions. We determined whether the binding pattern of MuSK IgG4 antibodies change throughout the disease course ("epitope spreading"), and affect disease severity or treatment responsiveness.

We mapped the MuSK epitopes of 255 longitudinal serum samples of 53 unique MuSK MG patients from three independent cohorts with ELISA.

Antibodies against the MuSK Iglike-1 domain determine disease severity. Epitope spreading outside this domain did not contribute to disease severity nor to pyridostigmine responsiveness. This provides a rationale for epitope specific treatment strategies.

INTRODUCTION

MuSK myasthenia gravis (MG) is caused by antibodies to the receptor tyrosine kinase MuSK at the neuromuscular junction (Klooster et al. 2012;McConville et al. 2004;Niks et al. 2008). Unique to the disease are the prevalent IgG4 MuSK antibodies that prevent MuSK-Lrp4 interactions in a complement-independent manner and lead to functional inhibition of the AChR clustering pathway (Huijbers et al. 2013;Koneczny et al. 2013;Mori et al. 2012). The extracellular domain of MuSK consists of three N-terminal Ig-like domains and a Frizzled-like domain (MuSK-Fz-like). Most patients carry antibodies to the Ig-like domain 1 (MuSK-Ig1), which contains residue I96 essential for MuSK-Lrp4 interaction (Zhang et al. 2011). Antibodies to MuSK-Ig1 are likely to inhibit either by physically obstructing MuSK-Lrp4 binding, or by changing the conformation of MuSK rendering it unable to interact with Lrp4 and other interacting proteins. Antibodies to the Ig-like 2 domain (MuSK-Ig2) and MuSK-Fz-like have also been described, but their role in the disease process is unclear (Huijbers et al. 2013;McConville, Farrugia, Beeson, Kishore, Metcalfe, Newsom-Davis, & Vincent 2004;Ohta et al. 2007). Moreover, intermolecular epitope spreading has been reported involving antibodies against MuSK and Lrp4, AChR or agrin (Gasperi et al. 2014;Higuchi et al. 2011;Zhang et al. 2012). Intramolecular and intermolecular epitope spreading has previously been described in bullous pemphigus where it correlated with disease severity (Di et al. 2011). Whether this is the case for MuSK MG is not known. Responsiveness to treatment with acetylcholine esterase inhibitor (AChEi) varies in MuSK MG. In AChR MG this treatment results in improvement of the symptoms by preventing breakdown of ACh. Thirty-fifty percent of MuSK MG patients treated with AChEi experience cholinergic side effects, ranging from cramps to worsening of symptoms (Evoli and Padua 2013). The AChE-ColQ complex is stabilized in the neuromuscular junction by interactions with MuSK and could be blocked by MuSK antibodies (Kawakami et al. 2012). Therefore, we hypothesized that increased AChEi sensitivity might be correlated with a specific epitope pattern of MuSK antibodies (Cartaud et al. 2004;Otsuka et al. 2015).

To investigate epitope spreading and the association with disease severity, reactivity patterns and treatment responsiveness in MuSK MG, we mapped and independently confirmed the epitopes for a large set of (longitudinal) serum samples from 53 patients.

PATIENTS AND METHODS

Patient material

Patients were retrospectively selected based on clinical weakness typical for MuSK MG and a positive MuSK RIA assay (RSR Ltd., Cardiff, UK) and the availability of longitudinal serum samples. The patients were followed at the Leiden University Medical Centre (LUMC), the University Medical Centre Groningen, the Hospital Santa Creu i Sant Pau in Barcelona or the Università Cattolica del Sacro Cuore in Rome.

The control group consisted of six healthy individuals, eight patients with Lambert-Eaton myasthenic syndrome (LEMS), and nine patients with seronegative MG. All patients and controls gave written informed consent and the study was approved by the LUMC medical ethical committee.

Severity of symptoms was evaluated retrospectively by experienced neurologists (JV, JK, EN, and II) using the disease severity score (DSS) (Niks et al. 2008). Neurologists were blinded for MuSK antibody titres and used information from patients' charts to evaluate the severity of symptoms on the date of each serum sample.

Cloning of target genes and recombinant protein purification

The coding region of nine MuSK protein fragments were amplified from full length human *MuSK* cDNA using primers containing *Nde*I and *Xho*I restriction sites (Supplementary table 1). The *MuSK* containing inserts were *Nde*I and *Xho*I digested and cloned into the pET28a vector (EMD Biosciences, Novagen Brand, Madison, WI). All vectors were sequence verified and were used to produce partially overlapping recombinant MuSK protein fragments (Supplementary table 2).
Protein production was performed as described previously (Huijbers et al. 2013).

Epitope mapping MuSK ELISA

Insoluble protein fragments were diluted in 1M urea and soluble protein fragments were diluted in PBS to a concentration of 3 µg/ml. 96-wells Maxisorp plates (Thermo Scientific, Nunc, Roskilde, Denmark) were coated with 100 µl diluted protein per well, and incubated overnight at 4 °C. After overnight incubation, the plate was processed as described previously (Huijbers et al. 2013).

Each ELISA experiment also included two negative control serum samples and one coating control per six plates to control for inter-plate and inter-experimental differences. As internal positive control, each plate contained a duplicate reactivity test for the full-length extracellular MuSK protein with a standard MuSK MG patient serum. All samples were tested in duplicate.

Statistical analysis

Each duplicate was averaged and corrected for the average PBS background signal. Each optical density value was next corrected for the internal positive control value. The 23 negative controls were used to determine the average background level. Signal detected in patients above this average background level plus three times the standard deviation were considered positive.

For statistical analysis the data was analysed using IBM SPSS statistics version 20 (SPSS Inc., Chicago, IL, U.S.A.). To assess the association between DSS and reactivity levels to MuSK-Ig1, taking into account the correlation between repeated measurements within patients, we fitted a linear mixed model with a fixed effect for the MuSK-Ig1 reactivity and random slopes and intercepts per patient. To

address whether there was additional effect of reactivity against other domains on disease severity we entered them separately into the model together with the MuSK-Ig1 variable.

RESULTS

Patient characteristics

To study epitope spreading in MuSK MG, 233 longitudinal serum samples of 20 Dutch and 11 Spanish patients were studied for their immunoreactivity against partially overlapping domains of human MuSK. Moreover, 22 samples of Italian patients were included to confirm our findings and study AChEi sensitivity in a separate cohort. Table 1 gives an overview of the demographic features of the patients included in this study. Mean age at onset was 42 years (49.2 in the Dutch population, 40.4 in the Spanish population, and 34.5 in the Italian cohort). The average age at onset in females was 8.89 years earlier compared to males although this difference was not significant (p=0.335). Average follow-up for the Dutch patients was 6.1 years (1.02 to 19.17). Mean follow up among men was 6.52 yrs (1.02-19.17) and for women 5.67 yrs (1.52-11.05) with substantial variation between patients (Table 1, Fig. 1A).

Table 1. Overview of demographic and clinical features of the patients included in this study.

	Dutch (n = 20)	**Spanish (n = 11)**	**Italian (n=22)**
Age at onset (range)	18.7 – 80.7	14 – 65	13-61
Sex F:M	10:10	8:3	16:6
Average follow-up in years (range)	5.8 (1.02-19.2)	5.07 (0-7.9)	-
Average number of included samples (range)	10.5 (3-21)	2.9 (1-4)	1
Mestinon at any point during disease	7	5	18
Azathioprine at any point during disease	14	2	12
Prednisone at any point during disease	17	8	19
Rituximab at any point during disease	0	6	2
IVIG at any point during disease	4	3	2
Plasmapheresis at any point during disease	6	0	10
Thymectomy	5	2	4
Co-morbidity	Diabetes mellitus type II: 3 Psoriasis: 1	-	Thyroiditis: 1 CIDP: 1

Epitope spreading is uncommon in MuSK myasthenia gravis

We defined epitope spreading as: 'the occurrence of reactivity to other epitopes in any of the serum samples of a patient compared to the reactivity pattern in the first available serum sample of this patient'. All Dutch and Spanish patients (n=31) showed reactivity to MuSK-Ig1 at the time of diagnosis. Sixteen patients showed additional

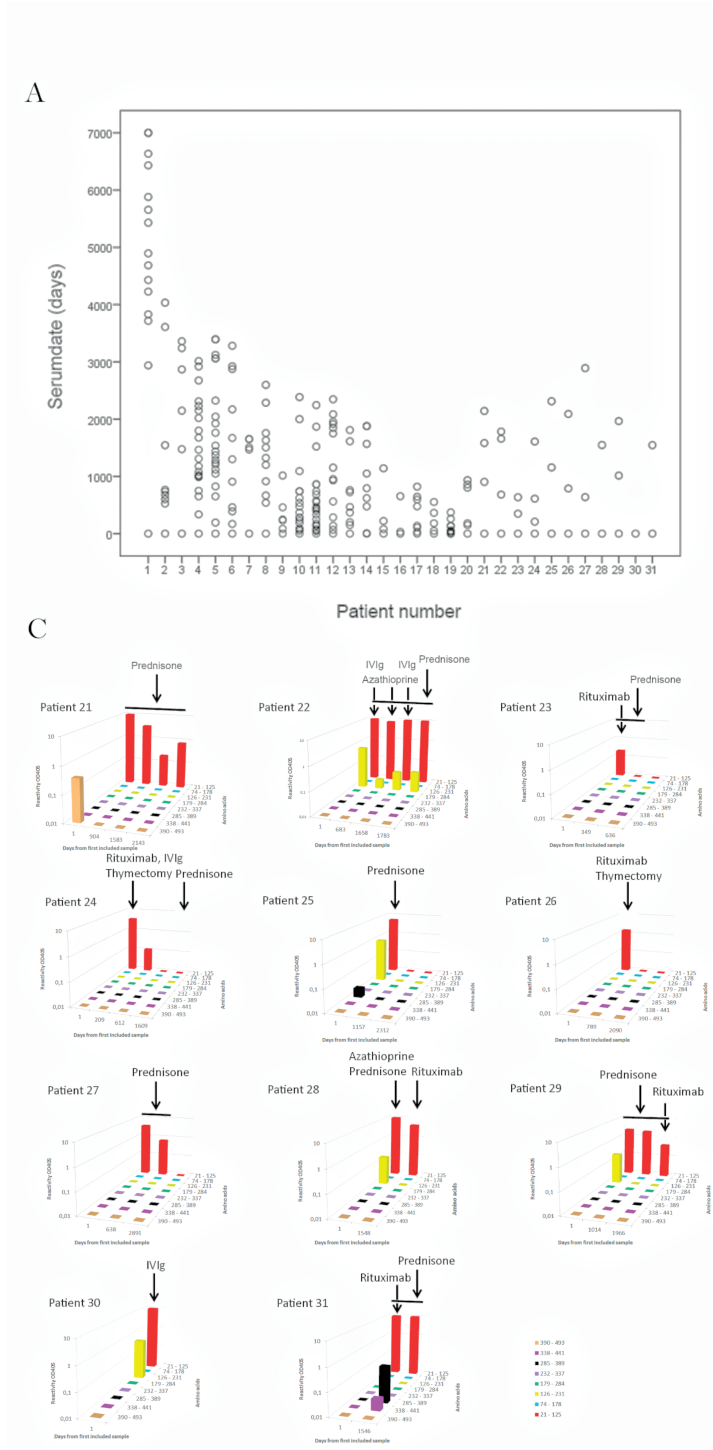
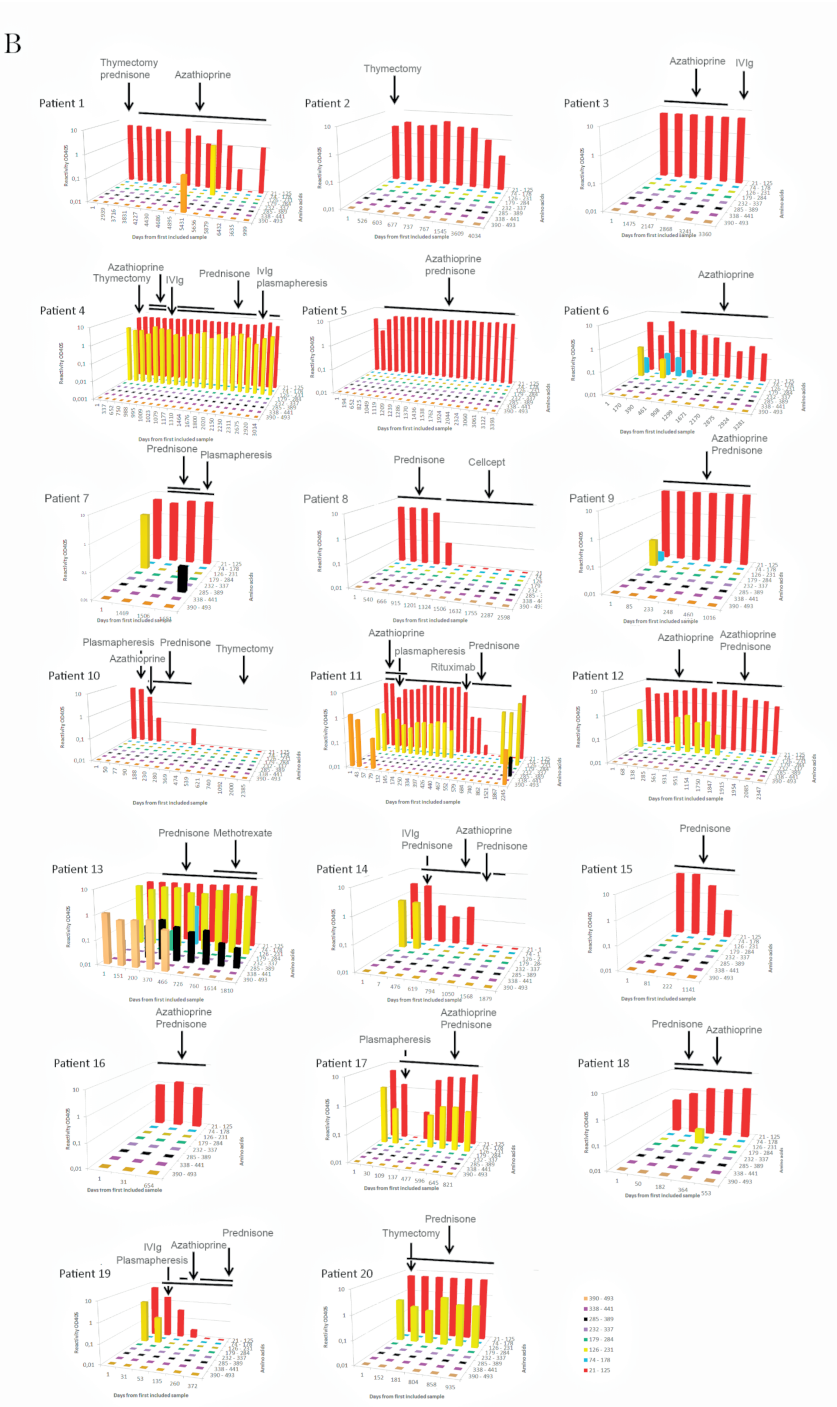


Figure 1. Overview of follow up and longitudinal epitope mapping patterns for 31 MuSK MG patients. Panel A shows the distribution of samples over the time of follow up for each patient. Reactivity patterns to the different MuSK proteins over time per patient reveal that epitope spreading is relatively uncommon (B Dutch patients, C Spanish patients). All patients show reactivity to ▸



the N-terminal MuSK protein domain. Reactivity to the MuSK-Ig2 is observed at any point during disease in 18 patients. The lines above each graph indicate the relevant treatment. Thymectomy had been performed in 6 patients previously to the discovery of MuSK antibodies in 2001.

reactivity to the MuSK-Ig2 and four patients had antibodies to the MuSK-Fz-like domain in the first available serum sample. In subsequent sera, epitope spreading was observed in 6 out of 31 patients accounting for 19% of MuSK MG patients tested (patients 1, 7, 11, 13, 18 and 31). When epitope spreading occurred, the majority of them developed reactivity against the MuSK-Fz-like domain (Fig. 1B, C). Three of these patients (7, 11, 13) already had reactivity against MuSK-Ig2 at the first time of examination, of which two (11 and 13) also had autoantibodies against the MuSK-Fz-like domain.

Of the patients who did not develop epitope spreading, 11 of 25 (44%) had only reactivity against MuSK-Ig1 (amino acids 21-125) whereas 48% also had reactivity against MuSK-Ig2 in their first available sample. Only two patients (8%) had reactivity against either the MuSK-Ig3 or the MuSK-Fz-like domain in addition to MuSK-Ig1 reactivity. None of the patients had reactivity against the intracellular domain at any point during their illness (data not shown).

Fig. 1 also illustrates the timing of the various treatments in the individual patients. As the treatment paradigms differed strongly between the patients it was not possible to statistically assess the effect of the treatments on reactivity against the different domains of MuSK. However, on the individual level the effects of treatment on antibody titres can be observed. Moreover, in five Italian patients, who went into remission, no reactivity against the MuSK-Ig1 domain could be detected, suggesting that these titres reflect their clinical status.

MuSK MG disease severity correlates with immunoreactivity against MuSK-Ig1 longitudinally

Since epitopes have been considered crucial determinants of the effectiveness and pathogenicity of an auto-immune response, we assessed whether reactivity against any domain of MuSK corresponded with the course of the disease and severity of the symptoms. A linear mixed effect model confirmed that reactivity against the N-terminal part of MuSK significantly correlates with DSS (combined cohorts: mean β-coefficient 0.159, $p<0.000002$, Dutch cohort: β-coefficient 0.175, $p<0.0001$, Spanish cohort: β-coefficient 0.107, $p<0.036$). This analysis took into account the individual correlation of each patient. This observation was subsequently confirmed in a third cohort of Italian patients (β-coefficient 0.167, $p< 0.026$). The average correlation between DSS and reactivity against MuSK-Ig1 for all patients is shown in Fig. 2A and for the individual cohorts in supplementary Fig.1. When including gender, age or treatment regimen in the mixed effect model this did not affect the association. Reactivity to other domains of MuSK did not contribute to disease severity, after correcting for the level of MuSK-Ig1 reactivity. Table 2 gives an overview of the significance of the correlation between the DSS and additional reactivity against the different domains of MuSK. In conclusion, antibodies against epitopes outside the N-terminal Ig-like 1 domain, and thus epitope spreading, do not seem to contribute to disease severity in our cohorts.

MuSK-Ig1 reactivity positively predicts disease severity between patients

Next, we investigated whether titres measured by our MuSK-Ig1 ELISA correlated more strongly to DSS than the values obtained by using the standard diagnostic RIA assay for MuSK MG, which is based on reactivity to the complete extracellular domain of MuSK. To address this we took the first samples of all our MuSK MG patients (Dutch and Spanish cohort) and established their DSS and their ELISA reactivity against MuSK-Ig1. When using a linear regression analysis the β-coefficient was 0.2882 (p=0.0013) (Fig.2B). When performing this test for the first available RIA value and corresponding DSS score for each patient (Fig.2C), the MuSK RIA values did not correlate with disease severity between patients (slope=0.322, p=0.083).

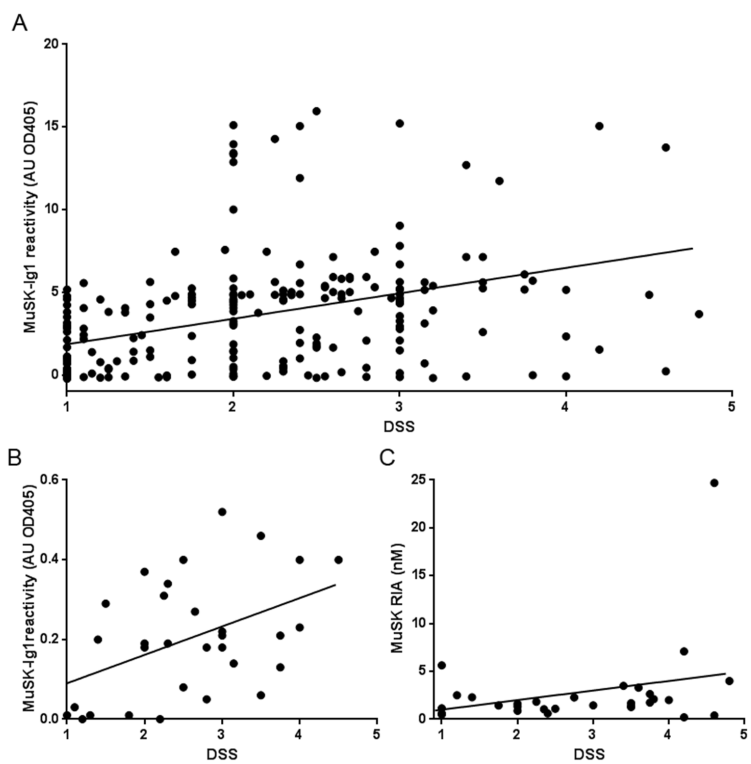


Figure 2. Overview of the average correlation between DSS and reactivity against the N-terminal domain for 53 MuSK MG patients (A). The multiple measures of each patient are represented by the dots. The correlation between the DSS and MuSK-Ig1 reactivity (B) or MuSK RIA results (C) from the first time point of each individual patient.

Reactivity patterns to MuSK differ between male and female patients

The data also allowed for the comparison of reactivity patterns with other demographic features of the three cohorts. Patients were stratified based on the maximum reactivity pattern. Thus when a patient at any point had reactivity against all MuSK Ig-like

Table 2. Overview of the significance level of each of the analysed MuSK proteins correlating to disease severity when reactivity to the MuSK-Ig1 is included as a covariate.

Protein fragment	p-value	β-coefficient	95% Confidence Interval
N-terminal Ig-like 1 MuSK 21-125	0.000002	0.157	0.108-0.204
MuSK 74-178	0.258	-0.77	-2.10-0.57
MuSK 126-231	0.065	0.40	-0.31-0.82
MuSK 179-284	0.326	-1.37	-4.27-1.54
MuSK 232-337	0.110	-2.00	-4.45-0.45
MuSK 285-389	0.848	-0.13	-1.51-1.24
MuSK 338-441	0.061	-2.51	-5.13-0.119
MuSK 390-493	0.278	0.53	-2.29-3.31

domains and the MuSK-Fz-like domain, even if this was only detected in a single sample, the patient was categorised in the Ig1+Ig2+Ig3/Fz group. Surprisingly, this distribution was significantly different between males and females (Fig. 3). Females more often had a restricted immune response against MuSK-Ig1 only, whereas all, but three, males had a broader immune response with at least antibodies to one additional protein fragment (Pearson Chi-Square p=0.039). This difference was not caused by variation in age at onset or duration of follow up.

Epitope patterns do not predict AChEi responsiveness

To investigate whether the presence of antibodies against other domains of MuSK correlate with treatment effects of AChEi we studied 14 Dutch and 18 Italian patients.

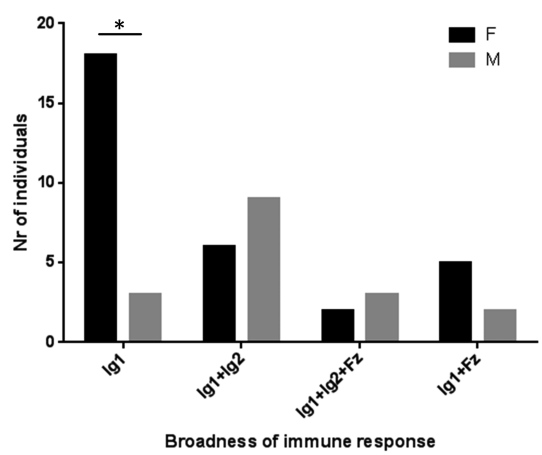


Figure 3. Reactivity patterns differ between male and females. Female MuSK MG patients have significantly more reactivity restricted to the Ig-like domain 1 compared to men.

When judging the antibody response to the maximal number of protein fragments, epitope patterns did not correlate with treatment responsiveness (Pearson Chi-square p=0.232). Also when patients were separated in two groups having either only MuSK-Ig1 domain antibodies or having a broader response to MuSK, this immune response did not correlate with a beneficial effect of AChEi (p=0.06). The well-known side effects of AChEi also did not correlate with the broadness of the anti-MuSK response (Pearson Chi-square test: p= 0.580). Moreover, as epitope patterns differ between males and females we investigated whether sex correlates with treatment responsiveness. Both sexes were distributed equally among the groups of responders and patients with side effects (Pearson Chi-square test: benefit p=0.948, side effects p=0.283). This data suggests that AChEi effectiveness and hypersensitivity are not predicted by sex nor the epitope specificity of the immune response against MuSK.

DISCUSSION

We here show that disease severity positively correlates with immunoreactivity against the N-terminal Ig-like domain of MuSK. This domain is crucial for interaction with Lrp4, in mediating the dimerization of the Agrin/Lrp4/MuSK heterotrimer complexes, MuSK activation/phosphorylation, and ultimately for AChR clustering and NMJ maintenance. This supports the observation that the main mode of action of MuSK IgG4 antibodies is interference with Lrp4 MuSK signalling (Huijbers et al. 2013, Otsuka et al. 2015). The importance of MuSK-Ig1 as the MIR of MuSK is supported by the observation that epitope spreading is uncommon and reactivity to other domains does not seem to contribute to a more severe disease outcome. This is different from other studies suggesting that epitope spreading is a beneficial process distracting the immune response away from the pathogenic epitope (Vincent et al. 1998)Others suggest that epitope spreading occurs early in the disease and significantly worsens the clinical outcome (Di et al 2011). We cannot exclude that the epitope spreading occured at an earlier or later disease stage. However, the epitope specificity appeared rather confined and stable during the disease course in the majority of MuSK MG patients over a period of minimally 5 years. Taken together these observations provide a rationale for using MIR (i.e. MuSK-Ig1) specific interventions for the treatment of MuSK MG.

In MuSK MG there appears to be a limited role for IgG1/IgG3 mediated structural damage of the neuromuscular synapse as seen in AChR antibody mediated MG (Engel and Arahata 1987). In AChR MG IgG1 or IgG3 mediated damage to the synapse is thought to expose the complete AChR leading to the generation of secondary reactivity against intracellular epitopes (Di et al. 2010;Di et al. 2011). In our cohorts of MuSK MG patients we found no reactivity against intracellular MuSK domains (data not shown). This corroborates on the observation that MuSK antibodies do not cause extensive physical breakdown of the NMJ or local inflammatory response, but rather disturb AChR clustering by preventing the AChR clustering signalling cascade (Ghazanfari et al. 2014;Klooster and Plomp et al. 2012;Mori et al.2012). It might also

suggest that the autoimmune response in MuSK is not the causative antigen that is presented in total to elicit the initial immune response, but that another antigen primes the immune system and induces crossreactive antibodies. If antigenic mimicry has a role in the initiation of MuSK MG, the MuSK-Ig1domain is the obvious candidate to search for crossreactive epitopes.

In autoimmune disease absolute antibody titres often do not predict disease severity, while within serum samples from a single MuSK MG patient a correlation between the antibody titre and disease severity can be observed (Niks et al. 2008). Indeed, within patients immunoreactivity against the MuSK extracellular domain often corresponds with clinical status (Bartoccioni et al. 2006). A decrease in antibody titre coincided with remission of the clinical features. In our cohort disease severity correlated well with the ELISA testing for MuSK-Ig1 reactivity both within and among patients. This suggests that the titre of antibodies against MuSK-Ig1 is a good predictor of disease severity.

The functional effects of ColQ and biglycan binding to MuSK are unknown (Amenta et al. 2012;Cartaud, Strochlic, Guerra, Blanchard, Lambergeon, Krejci, Cartaud, & Legay 2004). Both biglycan and ColQ interact with the MuSK-Ig1and the MuSK-Fz-like domain (Amenta et al. 2012, Otsuka et al. 2015). It would be interesting to explore whether the loss of these protein interactions by MuSK patient antibodies is relevant to the disease. One study has shown a dose-dependent loss of MuSK-ColQ interaction when exposed to MuSK antibodies derived from an active immunization model in rabbits (Kawakami et al. 2011). In line with this some have hypothesized that the AChEi hypersensitivity observed in many MuSK MG patients might be the result of loss of this interaction. In our study we could not confirm the occurrence of epitope dependent AChEi effectivity or hypersensitivity.

The MuSK-Fz-like domain functions as a Wnt receptor (Wu et al. 2010). Antibodies against this domain are seen in 22.6% of our MuSK MG patients. This is in concordance with a Japanese cohort were 30% of patients were shown to have antibodies against the MuSK-Fz-like domain (Takamori et al. 2013). Our study did not find a positive correlation between MuSK-Fz-like domain reactivity and disease severity. Perhaps the levels of MuSK-Fz-like domain antibodies were too low, or do not interfere with Wnt signalling. Although geographic effects in MuSK autoimmunity and higher involvement in Asians (Suzuki et al. 2011) suggest a genetic and/or environmental contribution to the development of MuSK autoimmunity, the epitope data available from three different European cohorts and a Japanese cohort does not support population differences in the immunoreactivity pattern.

One of the more striking observations in our study is the difference between men and women in their immune reactivity against MuSK. An antibody response restricted to the MuSK-Ig1 domain was almost exclusively found in women. Sera from men recognize a larger number of MuSK epitopes than sera from female MG patients. Although we do not have an explanation for this finding, it seems robust, as it was present in all three cohorts of patients that were studied. The three male patients,

with a restricted MuSK antibody profile, all had a relatively low titer. Also, epitope spreading in these patients might have been missed due to the lack of follow-up sera in these patients. Differences in age at onset or follow up time did not explain the differences in the reactivity pattern.

In conclusion, epitope spreading occurs in a minority of the MuSK MG patients. The correlation between MuSK Ig1-like domain reactivity with disease severity indicates that blocking of the Lrp4 MuSK interaction is a key factor in developing myasthenic weakness in MuSK MG.

ACKNOWLEDGEMENTS

We would like to thanks Ingrid Hegeman for providing us with excellent support in serum maintenance. This study was supported by funding of the Prinses Beatrix Spierfonds and the Association Française contre les Myopathies.

REFERENCES

1. Amenta, A.R., Creely, H.E., Mercado, M.L., Hagiwara, H., McKechnie, B.A., Lechner, B.E., Rossi, S.G., Wang, Q., Owens, R.T., Marrero, E., Mei, L., Hoch, W., Young, M.F., McQuillan, D.J., Rotundo, R.L., & Fallon, J.R. 2012. Biglycan is an extracellular MuSK binding protein important for synapse stability. *J.Neurosci.*, 32, (7) 2324-2334 available from: PM:22396407

2. Bartoccioni, E., Scuderi, F., Minicuci, G.M., Marino, M., Ciaraffa, F., & Evoli, A. 2006. Anti-MuSK antibodies: correlation with myasthenia gravis severity. *Neurology*, 67, (3) 505-507 available from: PM:16894117

3. Cartaud, A., Strochlic, L., Guerra, M., Blanchard, B., Lambergeon, M., Krejci, E., Cartaud, J., & Legay, C. 2004. MuSK is required for anchoring acetylcholinesterase at the neuromuscular junction. *J.Cell Biol.*, 165, (4) 505-515 available from: PM:15159418

4. Di, Z.G., Calabresi, V., Olasz, E.B., Zambruno, G., & Yancey, K.B. 2010. Sequential intramolecular epitope spreading of humoral responses to human BPAG2 in a transgenic model. *J.Invest Dermatol.*, 130, (4) 1040-1047 available from: PM:19812601

5. Di, Z.G., Thoma-Uszynski, S., Calabresi, V., Fontao, L., Hofmann, S.C., Lacour, J.P., Sera, F., Bruckner-Tuderman, L., Zambruno, G., Borradori, L., & Hertl, M. 2011. Demonstration of epitope-spreading phenomena in bullous pemphigoid: results of a prospective multicenter study. *J.Invest Dermatol.*, 131, (11) 2271-2280 available from: PM:21697892

6. Engel, A.G. & Arahata, K. 1987. The membrane attack complex of complement at the endplate in myasthenia gravis. *Ann.N.Y.Acad.Sci.*, 505, 326-332 available from: PM:3318619

7. Evoli, A. & Padua, L. 2013. Diagnosis and therapy of myasthenia gravis with antibodies to muscle-specific kinase. *Autoimmun.Rev.*, 12, (9) 931-935 available from: PM:23535158

8. Gasperi, C., Melms, A., Schoser, B., Zhang, Y., Meltoranta, J., Risson, V., Schaeffer, L., Schalke, B., & Kroger, S. 2014. Anti-agrin autoantibodies in myasthenia gravis. *Neurology*, 82, (22) 1976-1983 available from: PM:24793185

9. Ghazanfari, N., Morsch, M., Tse, N., Reddel, S.W., & Phillips, W.D. 2014. Effects of the ss2-adrenoceptor agonist, albuterol, in a mouse model of anti-MuSK myasthenia gravis. *PLoS.One.*, 9, (2) e87840 available from: PM:24505322

10. Higuchi, O., Hamuro, J., Motomura, M., & Yamanashi, Y. 2011. Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis. *Ann.Neurol.*, 69, (2) 418-422 available from: PM:21387385

11. Huijbers, M.G., Lipka, A.F., Plomp, J.J., Niks, E.H., van der Maarel, S.M., & Verschuuren, J.J. 2014. Pathogenic immune mechanisms at the neuromuscular synapse: the role of specific antibody-binding epitopes in myasthenia gravis. *J.Intern.Med.*, 275, (1) 12-26 available from: PM:24215230

12. Huijbers, M.G., Zhang, W., Klooster, R., Niks, E.H., Friese, M.B., Straasheijm, K.R., Thijssen, P.E., Vrolijk, H., Plomp, J.J., Vogels, P., Losen, M., van der Maarel, S.M., Burden, S.J., & Verschuuren, J.J. 2013. MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. *Proc.Natl.Acad.Sci.U.S.A*, 110, (51) 20783-20788 available from: PM:24297891

13. Kawakami, Y., Ito, M., Hirayama, M., Sahashi, K., Ohkawara, B., Masuda, A., Nishida, H., Mabuchi, N., Engel, A.G., & Ohno, K. 2011. Anti-MuSK autoantibodies block binding of collagen Q to MuSK. *Neurology*, 77, (20) 1819-1826 available from: PM:22013178

14. Klooster, R., Plomp, J.J., Huijbers, M.G., Niks, E.H., Straasheijm, K.R., Detmers, F.J., Hermans, P.W., Sleijpen, K., Verrips, A., Losen, M., Martinez-Martinez, P., De Baets, M.H., van der Maarel, S.M., & Verschuuren, J.J. 2012. Muscle-specific kinase myasthenia gravis IgG4 autoantibodies cause severe neuromuscular junction dysfunction in mice. *Brain*, 135, (Pt 4) 1081-1101 available from: PM:22396395

15. Koneczny, I., Cossins, J., Waters, P., Beeson, D., & Vincent, A. 2013. MuSK myasthenia gravis IgG4 disrupts the interaction of LRP4 with MuSK but both IgG4 and IgG1-3 can disperse preformed agrin-independent AChR clusters. *PLoS.One.*, 8, (11) e80695 available from: PM:24244707

16. McConville, J., Farrugia, M.E., Beeson, D., Kishore, U., Metcalfe, R., Newsom-Davis, J., & Vincent, A. 2004. Detection and characterization of MuSK antibodies in seronegative myasthenia gravis. *Ann.Neurol.*, 55, (4) 580-584 available from: PM:15048899

17. Mori, S., Kubo, S., Akiyoshi, T., Yamada, S., Miyazaki, T., Hotta, H., Desaki, J., Kishi, M., Konishi, T., Nishino, Y., Miyazawa, A., Maruyama, N., & Shigemoto, K. 2012. Antibodies against muscle-specific kinase impair both presynaptic and postsynaptic functions in a murine model of myasthenia gravis. *Am.J.Pathol.*, 180, (2) 798-810 available from: PM:22142810

18. Niks, E.H., van, L.Y., Leite, M.I., Dekker, F.W., Wintzen, A.R., Wirtz, P.W., Vincent, A., van Tol, M.J., Jol-van der Zijde CM, & Verschuuren, J.J. 2008. Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1. *J.Neuroimmunol.*, 195, (1-2) 151-156 available from: PM:18384886

19. Ohta, K., Shigemoto, K., Fujinami, A., Maruyama, N., Konishi, T., & Ohta, M. 2007. Clinical and experimental features of MuSK antibody positive MG in Japan. *Eur.J.Neurol.*, 14, (9) 1029-1034 available from: PM:17718696

20. Otsuka, K., Ito, M., Ohkawara, B., Masuda, A., Kawakami, Y., Sahashi, K., Nishida, H., Mabuchi, N., Takano, A., Engel, A.G., & Ohno, K. 2015. Collagen Q and anti-MuSK autoantibody competitively suppress agrin/LRP4/MuSK signaling. *Sci.Rep.*, 5, 13928 available from: PM:26355076

21. Suzuki, S., Utsugisawa, K., Nagane, Y., Satoh, T., Kuwana, M., & Suzuki, N. 2011. Clinical and immunological differences between early and late-onset myasthenia gravis in Japan. *J.Neuroimmunol.*, 230, (1-2) 148-152 available from: PM:21074862

22. Takamori, M., Nakamura, T., & Motomura, M. 2013. Antibodies against Wnt receptor of muscle-specific tyrosine kinase in myasthenia gravis. *J.Neuroimmunol.*, 254, (1-2) 183-186 available from: PM:22999188

23. Vincent, A., Willcox, N., Hill, M., Curnow, J., MacLennan, C., & Beeson, D. 1998. Determinant spreading and immune responses to acetylcholine receptors in myasthenia gravis. *Immunol.Rev.*, 164, 157-168 available from: PM:9795773

24. Wu, H., Xiong, W.C., & Mei, L. 2010. To build a synapse: signaling pathways in neuromuscular junction assembly. *Development*, 137, (7) 1017-1033 available from: PM:20215342

25. Zhang, B., Tzartos, J.S., Belimezi, M., Ragheb, S., Bealmear, B., Lewis, R.A., Xiong, W.C., Lisak, R.P., Tzartos, S.J., & Mei, L. 2012. Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. *Arch.Neurol.*, 69, (4) 445-451 available from: PM:22158716

26. Zhang, W., Coldefy, A.S., Hubbard, S.R., & Burden, S.J. 2011. Agrin binds to the N-terminal region of Lrp4 protein and stimulates association between Lrp4 and the first immunoglobulin-like domain in muscle-specific kinase (MuSK). *J.Biol.Chem.*, 286, (47) 40624-40630 available from: PM:21969364

SUPPLEMENTARY DATA

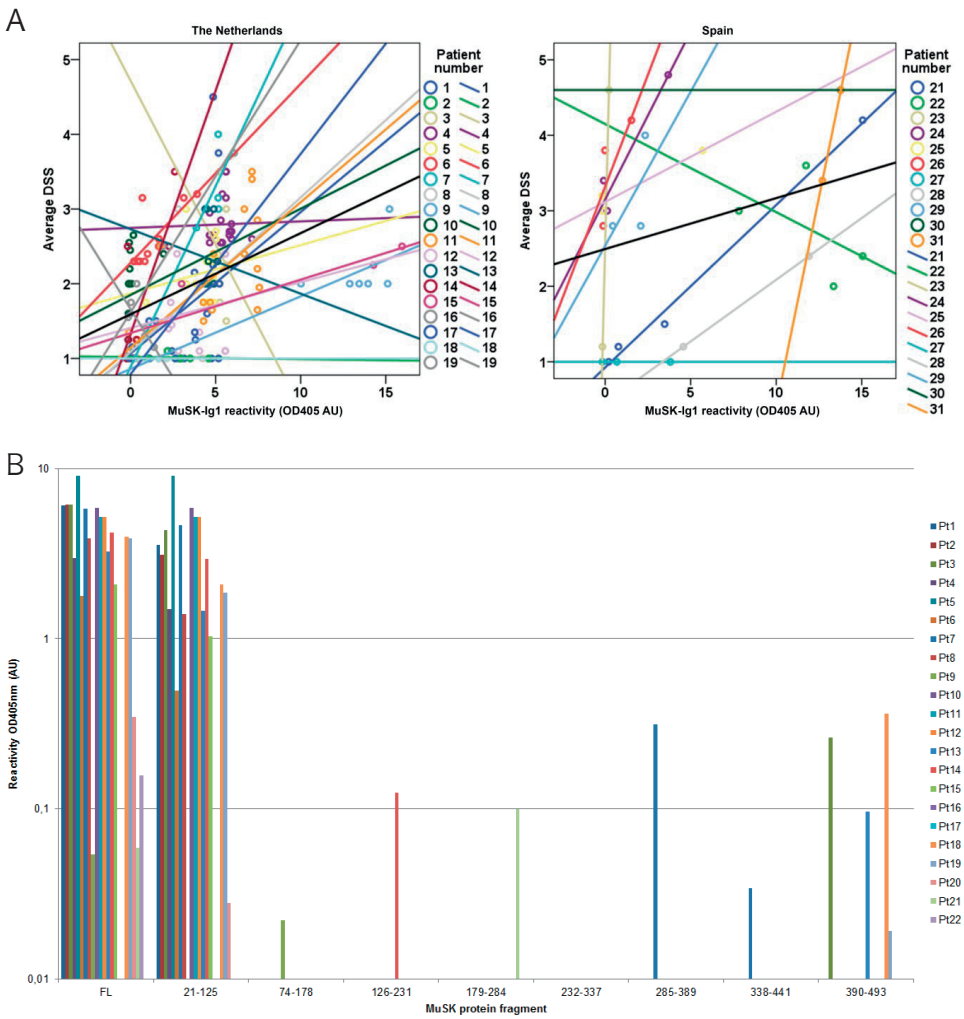


Figure S1. Overview of the association between DSS and MuSK-Ig1 reactivity for each patient and separated on origin (A) and of the epitope patterns of 22 Italian MuSK MG patients (B).

Table S1. Primer pairs for amplifying the different MuSK proteins DNA fragments.

Primer name	Sequence	
MuSK 21-125	Fw	5′ –GCA CAT ATG ACT GAG AAA CTT CCA AAA GCTC– 3′
	Rev	5′ –AG ATG AAA CCT AAA ATA ACT CGC TAG CTC GAG GC– 3′
MuSK 74-178	Fw	5′ –GCA CAT ATG CGG TAC AGC ATC CGG GAG A– 3′
	Rev	5′ –TCT GGG CGA TTG AGG ATT CAT TAG CTC GAG GCA– 3′
MuSK 126-231	Fw	5′ –GCA CAT ATG CCT CCC ATA AAT GTG AAA ATA ATA– 3′
	Rev	5′ –TT GGC TTT GTG ACC CTG TAG CTC GAG GCA– 3′
MuSK 179-284	Fw	5′ –GCA CAT ATG AAC GTA CAA AAG GAA GAT GCA G– 3′
	Rev	5′ –GGA CTC TAC ACA TGC GCG GCT TAG CTC GAG GCA– 3′
MuSK 232-337	Fw	5′ –GCA CAT ATG CAC TGT ACA GCA ACA GGC ATT– 3′
	Rev	5′ –CA AAA GAT GCT CTT GTT TTT CGC TAG CTC GAG GCA– 3′
MuSK 285-389	Fw	5′ –GCA CAT ATG ACC AAT AAG CAT GGG GAG AAG– 3′
	Rev	5′ –GT CCT GGA GTA GTG CCT ACT TAG CTC GAG GCA– 3′
MuSK 338-441	Fw	5′ –GCA CAT ATG AAC ACC TCC TAT GCG GAC C– 3′
	Rev	5′ –GC AAG CTT CCC AGC ATG CAT TAG CTC GAG GCA– 3′
MuSK 390-493	Fw	5′ –GCA CAT ATG CCT ATT CCC ATT TGC AGA GAG– 3′
	Rev	5′ –TCT GTC TCA CCT ACA TAC TCC TAG CTC GAG GCA– 3′
MuSK 441-773	Fw	5′ –GCA CAT ATG TCA GCA GCA GTA ACC CTC A – 3′
	Rev	5′ –CGC ATG TGT GAG AGG GCA CTC GAG GCA– 3′

Table S2. Overview of amino acid sequences of the recombinant MuSK proteins used in the ELISA assays

Recombinant protein	Amino acid sequence
MuSK 21-125	TEKLPKAPVITTPLETVDALVEEVATFMCAVESYPQPEISWTRNKILIKLFDTRYSIRENGQ LLTILSVEDSDDGIYCCTANNGVGGAVESCGALQVKMKPKITR
MuSK 74-178	RYSIRENGQLLTILSVEDSDDGIYCCTANNGVGGAVESCGALQVKMKPKITRPPINVKIIE GLKAVLPCTTMGNPKPSVSWIKGDSPLRENSRIAVLESGSLRIH
MuSK 126-231	PPINVKIIEGLKAVLPCTTMGNPKPSVSWIKGDSPLRENSRIAVLESGSLRIHNVQKEDAG QYRCVAKNSLGTAYSKVVKLEVEVFARILRAPESHNVTFGSFVTL
MuSK 179-284	NVQKEDAGQYRCVAKNSLGTAYSKVVKLEVEVFARILRAPESHNVTFGSFVTLHCTATGI PVPTITWIENGNAVSSGSIQESVKDRVIDSRLQLFITKPGLYTCIA
MuSK 232-337	HCTATGIPVPTITWIENGNAVSSGSIQESVKDRVIDSRLQLFITKPGLYTCIATNKHGEKFS TAKAAATISIAEWSKPQKDNKGYCAQYRGEVCNAVLAKDALVFL
MuSK 285-389	TNKHGEKFSTAKAAATISIAEWSKPQKDNKGYCAQYRGEVCNAVLAKDALVFLNTSYA DPEEAQELLVHTAWNELKVVSPVCRPAAEALLCNHIFQECSPGVVPT
MuSK 338-441	NTSYADPEEAQELLVHTAWNELKVVSPVCRPAAEALLCNHIFQECSPGVVPTPIPICREY CLAVKELFCAKEWLVMEEKTHRGLYRSEMHLLSVPECSKLPSMH
MuSK 390-493	PIPICREYCLAVKELFCAKEWLVMEEKTHRGLYRSEMHLLSVPECSKLPSMHWDPTACA RLPHLDYNKENLKTFPPMTSSKPSVDIPNLPSSSSSSFSVSPTYS
MuSK 441-773	SAAVTLTTLPSELLLDRLHPNPMYQRMPLLLNPKLLSLEYPRNNIEYVRDIGEGAFGRVF QARAPGLLPYEPFTMVAVKMLKEEASADMQADFQREAALMAEFDNPNIVKLLGVCAV GKPMCLLFEYMAYGDLNEFLRSMSPHTVCSLSHSDLSMRAQVSSPGPPPLSCAEQLCI ARQVAAGMAYLSERKFVHRDLATRNCLVGENMVVKIADFGLSRNIYSADYYKANENDA IPIRWMPPESIFL