Myasthenia gravis with antibodies to muscle-specific kinase

Clinical characteristics, epidemiology, and immunological aspects

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Cover image: parasternal intercostal muscle biopsy from a MuSK MG patient, showing increased branching of intramuscular nerves compatible with nerve sprouting.

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General introduction

Introduction to myasthenia gravis

Myasthenia gravis (MG) is one of the best characterised neuromuscular or autoimmune disorders. The Greek words myos (muscle) and asthenos (weakness) refer to the presence of pure motor weakness that can affect extraocular, bulbar, axial and limb muscles. Myasthenic weakness is characterised by its fluctuating nature and fatigability. When symptoms are restricted to ophthalmoparesis and ptosis, MG is classified as ocular MG (OMG). Weakness in other muscles is termed generalised MG (GMG). Thomas Willis was probably the first to describe a myasthenic patient in his book "De Anima Brutorum" from 1672. He described a "prudent and honest woman" suffering from fluctuating weakness in the arms and legs, but also from compromised articulation. [1]

> *"For some time she can speak freely and readily enough, but after long, hasty or laborious speaking she is not able to speak a word, but becomes mute as a fish and does not recover till after an hour or two"*

It cannot be proven, nor ruled out, that this was in fact the first patient described with autoantibodies to muscle-specific kinase (MuSK), one of the key elements in neuromuscular signal transmission, as will be highlighted in this thesis. By the end of the 19th century, Wilhelm Erb and Samuel Goldflam provided a detailed description of the disease in which ptosis and ophthalmoparesis frequently occur as first symptoms, followed by weakness of chewing, swallowing and speaking, with a further spread towards neck and limb muscles. The clinical hallmark of MG is the fluctuating character of the weakness, becoming worse upon sustained movement and by the end of the day. [2,3] In 1934, Mary Walker reported a temporary improvement of facial weakness in an MG patient after subcutaneous injections of physostigmine. By then, it was known that physostigmine inhibited the breakdown of the neurotransmitter acetylcholine (ACh). Together with the discovery of the role of ACh in neuromuscular signal transmission by Dale and Feldberg in that same year, the pathology of MG was thought to be a curare-like poisoning of the "motor end-organs or myoneural junctions". [4,5] The first description of histological malformation of the neuromuscular junction (NMJ) in MG patients was made by Coërs and Desmedt in 1959. [6] In 1960, after having studied 440 MG cases, the Scottish neurologist Simpson postulated the still current autoimmune hypothesis of the mechanism of MG, based on observations including thymic hyperplasia and occasional transfer of the disease from a mother to her newborn child during the first weeks of life. He also acknowledged a genetic susceptibility for immunological disorders such as systemic lupus erythematosus in patients and thyroid disease in their relatives. [7] In 1976, the biochemist Lindstrom induced antibodies as a coincidental side-effect of his studies in rabbits injected with the postsynaptic acetylcholinereceptor (AChR) derived

from electric eels. [8] The anti-AChR antibody positive and weak rabbits were recognised as being "myasthenic". The final step was made when Lindstrom *et al*. were able to identify autoantibodies to the AChR as the causative factor for this transmission defect in 87% of 71 MG patients. [9]

Pathogenesis of classical MG with autoantibodies to AChR

The AChR is a cation channel formed by five subunits. In human muscle, two subtypes occur *i.e.* a fetal receptor consisting of two α1 subunits and one β1, γ and δ subunit, and an adult receptor in which the γ subunit is replaced by an ε subunit. The receptors are densely concentrated and located at the crests of the folds of the postsynaptic muscle membrane. A nerve action potential causes ACh to be released from presynaptic vesicles, which then diffuses across the synaptic cleft. Binding of ACh to the AChR depolarizes the postsynaptic endplate membrane generating an endplate potential which then opens Na^+ channels located in the depths of the postsynaptic folds. This causes further depolarisation, finally triggering an action potential that propagates along the muscle fiber leading to muscle contraction. ACh diffuses out of the synaptic cleft or is readily hydrolysed by acetylcholinesterase (AChE) thus ending the depolarisation. The endplate potential generated by ACh release and binding of ACh to the AChR is normally much higher than the threshold needed to generate a muscle action potential. In myasthenic syndromes this safety factor is typically compromised, either by pre- or postsynaptic pathology.

In classical MG with autoantibodies to the AChR (AChR MG), the antibodies affect the neuromuscular transmission in three ways. Firstly, antibodies can directly compete with ACh binding, although titres of such blocking antibodies only weakly correlated with disease severity. [10] More importantly, antibodies are able to crosslink AChRs, thereby increasing AChR turnover through internalisation and lysosomal degradation by muscle fibers. [11]

However, the third and most crucial factor affecting transmission is a reduction in the number of available AChRs and changes in endplate morphology due to complement activation, as was shown by Lennon *et al.* in a rat model using passive and active immunisation. [12] Complement deposition was located at the neuromuscular junction in MG patients correlating with destruction of the folded postsynaptic membrane. [13,14] In 1987, Rodgaard *et al*. published the predominance of antigen-specific IgG subclasses 1 and 3 directed to AChR in MG patients. Those subclasses are well known for their complement activating capacity. [15]

The presence of other autoimmune diseases in patients with AChR MG and their first-degree relatives suggests a role for a genetic predisposition. The human leukocyte antigen (HLA) concerns large number of genes that encode proteins involved in the presentation of antigens to the immune system. An extremely large genetic variability of HLA molecules within

the human population is an important factor determining the flexibility of the acquired immune system. However, several HLA haplotypes are thought to increase the likelihood of developing autoimmune diseases. For example, the HLA A1-B8-DR3 ancestral haplotype is associated with many autoimmune diseases, including Graves' disease, [16] celiac disease, [17] the Lambert-Eaton myasthenic syndrome (LEMS) without small cell lung carcinoma, [18] and type I diabetes mellitus. [19] AChR MG without thymoma and an onset before the age of 40 years is also strongly associated with this haplotype in white Europeans. [20-23] By contrast, late onset MG which occurs more frequently in men has been linked to HLA DR2. [23-25]

Definition of "seronegative" MG

The discovery of antibodies to the AChR in the majority of MG patients in 1976 immediately defined a group of patients diagnosed as MG based on their phenotype in whom no such antibodies could be found. They were defined as "seronegative" MG (SNMG). Between 1976 and 1990, the prevalence of SNMG was reported in at least 12 studies. Overall SNMG was present in 48.1% of patients who had ocular symptoms only (weighted average of n = 412, range 21-83%) and 11.5% of patients with generalised weakness (weighted average of n = 2272, range 6-25%). [9,23,26-35] Although the lower frequency in GMG compared to OMG is consistently found, the large variability probably reflects patient selection and referral bias.
Multiple explanations for the absence of antibodies in the presence of a myasthenic phenotype were proposed including the possibility of seroconversion early in the course of the disease, [36] seroconversion after treatment with immunosuppressive therapy, [29,36,37] or during periods of clinical remission. [38] In children, seroconversion has been described occurring after many years, ranging from 5-10 years, after the onset of (ocular) symptoms. [39]
Adaptations to the assays used to detect AChR antibodies decreased the number of SNMG patients by using human AChR instead of rat or calf derived AChR, [40,41] a mixture of human AChR derived from normal and denervated muscle instead of denervated muscle only, [42] or AChR derived from cell lines. [43] This fine-tuning has continued in recent years through the introduction of cell-based assays using sensitive fluorescence techniques to detect low-affinity antibodies as will be highlighted in the Chapters 3 and 5 of this thesis. [44]
Finally, it became clear that congenital myasthenic syndromes caused by mutations in proteins involved in neuromuscular transmission such as rapsyn and Dok7 could also present as a late onset phenotype. [45,46]
However, strong evidence arose that SNMG was in fact an acquired autoimmune disease caused by autoantibodies directed to unknown antigens. Firstly, clinical symptoms responded to immunosuppressive therapy and plasmapheresis. [27,34,47,48] Secondly, the disease could be transferred from mother to child through the placenta. [49,50] Thirdly, passive transfer using

human serum or IgG from SNMG patients caused a neuromuscular transmission defect and possibly a small loss of AChRs in the mouse diaphragm. [51,52] Moreover, IgG from SNMG patients was shown to bind to an unknown antigen present on a muscle cell line. [53]

Autoantibodies to MuSK in seronegative myasthenia gravis

In 2001, Hoch *et al*. were the first to describe autoantibodies in 17 out of 24 patients with generalised SNMG directed to the extracellular domain of an antigen called muscle-specific kinase (MuSK) using immunofluorescence on transfected cells, immunoprecipitation and ELISA. The autoantibodies also interfered with MuSK function, *i.e.* they prevented the formation of AChR clusters normally induced by nerve-derived agrin. [54] Patients with MG who had autoantibodies to MuSK (MuSK MG) appeared to differ clinically from AChR antibody negative/MuSK antibody negative patients because weakness was more prominent in oculobulbar muscles with frequent respiratory insufficiency. [55] In the MuSK MG group, treatment with oral immunosuppressants seemed less effective. Some MuSK MG patients adversely responded to AChE inhibitors with widespread fasciculations, and muscle atrophy occurred later in the course of the disease. [56] In retrospect, this phenotype had already been identified as a subgroup within a cohort of SNMG patients by Evoli *et al*. in 1996. [57] Studying larger cohorts of AChR Ab- MG patients, the percentage of MuSK MG was adjusted downward leaving a significant group of AChR Ab-/MuSK Ab- patients nonetheless.

The role of MuSK in the neuromuscular junction

Synaptic development of a neuromuscular junction requires specialisation of muscle fibers in the proximity of a motor nerve terminal. This specialisation includes at least two mechanisms, *i.e.* the local transcription of specific proteins by the synaptic nuclei including the AChR subunits, and the redistribution of pre-existent molecules present on the surface of the myofiber. The result is clustering of AChRs on the crests of the postsynaptic membrane folds and the formation of postsynaptic intracellular signalling pathways.
Nerve-derived agrin, a 200 kDa heparin sulphate glycoprotein, proved able to aggregate AChR in cultured chicken myotubes, a process inhibited by polyclonal anti-agrin antibodies. [58] Because phosphotyrosine staining in myofibers is concentrated at the NMJ and tyrosine kinases are often involved in cell-cell interaction, Jennings *et al*. used polymerase chain reaction (PCR) to look for tyrosine kinases in the electric organ and skeletal muscle of the electric ray *Torpedo californica.* They were able to isolate a transmembrane tyrosine kinase with a size of 100 kDa. [59] In 1995, Valenzuela *et al*. cloned similar receptor protein kinases using

cDNA from rat muscle and both normal and denervated human muscles. They introduced the term muscle-specific kinase (MuSK). In this study, MuSK colocalised with AChR at the neuromuscular junction. MuSK expression was upregulated after fusion of myoblasts to form myotubes. In normal adult muscle, MuSK was present only at the neuromuscular junction, but MuSK and AChR transcripts became widely detectable throughout the myofibers after denervation. Activated MuSK dimerizes and triggers phosphorylation of the β subunit of the AChR. [60]

The *MuSK* gene contains 14 exons and maps to chromosome 9q in humans and to chromosome 4 in mice. [61] The extracellular domain of MuSK contains 4 immunoglobulin-like domains and a cystein rich domain. MuSK has a molecular weight of 110 kDa and contains a single transmembrane region followed intracellularly by a juxtamembrane domain containing one of the four tyrosine residues (Tyr553) that is first autophosphorylated upon activation by agrin (Figure 1.1). [62] Next is the catalytic domain containing the tyrosine kinase itself. This kinase contains three tyrosine autophosphorylation sites in an activation loop (Tyr750, 754 and 755). Such a loop is a general characteristic of the receptor tyrosine kinase family that autoregulates catalytic activity by obstructing adenosine triphosphate (ATP) and substrate binding. After phosphorylation of the tyrosine residues, MuSK is switched to a stable active state. [63] Activated MuSK is then rapidly internalised through endocytosis which is also a common property for receptor tyrosine kinases. [64]

Interactions with other proteins

Agrin

In 1996, DeChiara *et al*. reported on a mouse model carrying a *MuSK* gene disruption in which mice were immobile and died at birth. Their skeletal muscles were grossly normal, indicating normal early skeletal muscle development. However, pre- and postsynaptic differentiation was absent, and intramuscular nerves were not able to contact the muscle fibers. [65] Postpartum inhibition of MuSK by RNA interference also induced the disassembly of NMJs that had already been built, indicating also a role for MuSK in NMJ maintenance. [66] Mice lacking agrin showed a remarkably similar phenotype either dying in utero or being stillborn. [67] In 1996, Glass *et al*. reported that agrin was indeed the factor inducing the phosphorylation of MuSK. Even long-lasting phosphorylation of MuSK and AChR, as well as clustering, can be initiated be a single five minute pulse of agrin. [68] In 2006, Stiegler *et al.* showed that the most N-terminal Ig-like domain in MuSK is crucial for this function. [69]

LRP4

No direct binding of agrin to the MuSK ectodomain could be demonstrated. Phosphorylation of MuSK in response to agrin only took place in myotubes, but not in myoblasts or other cell types, transfected with a MuSK expression factor. This suggested the presence of another hypothetical component termed myotube associated specificity component (MASC). [70] Moreover, whereas the intracellular MuSK kinase domain was sufficient for MuSK autophosphorylation and AChR phosphorylation, the ectodomain of MuSK was essential for clustering of the AChRs. [71] In 2006, Weatherbee *et al*. showed that mice with a mutation in the gene encoding the low-density lipoprotein receptor-related protein 4 (LRP4) died at birth and their synapses had both pre- and postsynaptic defects very much like MuSK mutant mice. This included aberrant motor axon growth, axonal branching and a lack of postsynaptic clustering. [72] In 2008, the receptor for neural agrin proved to be LRP4 according to two independent groups. [73,74] LRP4 associates with itself and forms a complex with MuSK in the absence of agrin. LRP4 is expressed specifically in myotubes, but not myoblasts, thereby explaining its function as the above mentioned MASC. The intracellular domain of MuSK becomes tyrosine phosphorylated upon stimulation of LRP4 with agrin. The binding between LRP4 and agrin is enhanced approximately 20 fold by the presence of calcium. [73]

Rapsyn

Similar to MuSK, the intracytoplasmatic protein rapsyn (an acronym for receptor-associated protein of the synapse) was also known to colocalise with AChR at the developing NMJ. In 1995, Gautam *et al*. showed that rapsyn null mice died within hours after birth. There were no detectable AChR clusters anywhere along the muscle fibers which proved that rapsyn was essential for clustering. [75] Recently, the binding between AChR and rapsyn was found to depend on a 20 amino acid motif in the intracellular domain of the AChR β subunit in which one tyrosine amino acid (Tyr390) is phosphorylated through the agrin-MuSK pathway. Because rapsyn has also AChR binding capacity independent of the β loop, it is postulated that more than one rapsyn molecule can bind to phosphorylated AChR. Rapsyn thus acts as a scaffold protein in the NMJ. [76]

Dok7

The cytoplasmatic protein Dok7 (downstream of tyrosine kinase 7) also proved essential for NMJ formation. Okada *et al*. showed that Dok7 null mice died at birth and did not form AChR clusters or NMJs. [77]

The phosphorylated tyrosine in the juxtamembrane domain of MuSK mentioned earlier (Tyr553) becomes a docking site for the phosphotyrosine binding site of Dok7. A tetramer is then created in which a Dok7 dimer binds to two MuSK monomers (Figure 1.1). The juxtapositioning of two MuSK kinase domains facilitates the autophosphorylation of the

MuSK activation loop. Therefore Dok7 is not only a substrate for MuSK, but also activates the catalytic domain. [78] The binding of Dok7 to MuSK is also dependent on the interaction of MuSK and a tumorous imaginal disc protein (Tid1), which function remains to be clarified further. [79]

Acetylcholinesterase (AChE)

An important enzyme in limiting the duration of the presynaptic trigger by the released ACh is acetylcholinesterase (AChE). This enzyme is organised into 3 tetramers of catalytic subunits that are linked to collagen Q (ColQ), a triple helical collagen subunit. ColQ itself is connected to the postsynaptic basal lamina by binding to perlecan, but also to MuSK through its C-terminal domain. [80]

Intracellular interactions

The intracellular pathways leading from MuSK activation to a fully developed NMJ with stable AChR clusters and a sustained MuSK activation are becoming increasingly complex and remain poorly understood. It requires the presences of multiple intracytoplasmatic enzymes like geranylgeranyltransferase I and Rac I essential for the first micro aggregates, Rho GTPase and possibly PAK1 that are involved in the formation of larger clusters and non-receptor tyrosine kinases like Src and Abl that play a role in the early and late response to agrin. Multiple enzymes have been found to contribute to negative feedback pathways and are involved in the disassembly of AChR clusters. As mentioned earlier, activated MuSK is internalised through endocytosis. For this process, the ATPase N-ethylmaleimide sensitive fusion protein (NSF) that binds directly to MuSK is essential. [64] Internalised MuSK is probably degraded upon binding to the ubiquitin tag to direct it to the proteasome. In this respect, a PDZ domain containing RING finger 3 (PDZRN3) ligase has been identified that binds to MuSK and regulates MuSK cell surface levels. [81] MuSK also binds to caveolin 3, a structural protein component of caveolae in muscle cells, but the role of this interaction remains to be elucidated. [82] A delicate balance between these mechanisms probably guides the ongoing process of building and remodelling of NMJs. [83]

Role of MuSK prior to neural innervation

AChR clusters are also formed on immature muscle fibers *prior* to the innervation by motor nerves. In 2001, Lin *et al*. showed that in mice during embryogenesis at day 14.5 AChR clusters were present, but not apposed by a nerve terminal whereas at day 18.5 all remaining clusters were apposed by a nerve terminal. This process, called prepatterning, is restricted to the central part of the muscle fiber. However, no clustering took place in MuSK or rapsyn mutant models indicating that these two proteins are also essential in clustering prior to neural innervation. Agrin was found to be essential in a later stage for stabilisation and growth of the neurally apposed synapses and dispersion of aneural clusters. [84]

Yang *et al*. reported similar results in a mouse model in which motor neurons lose their ability to differentiate through inactivation of the homeodomain transcription factor HB9. AChRs still cluster in these mice, and AChR transcription is increased in the central region of the diaphragm muscle, although in a wider zone than normal. For this clustering, postsynaptic MuSK, but not nerve-derived agrin, proved to be essential. [85] In 2008, Kim and Burden showed that MuSK expression itself is patterned in this central region of the muscle fiber in the absence of neural innervation, and that ectopic MuSK expression induces ectopic AChR clusters promoting axonal outgrowth. [86] Recently, a key protein in this pre-patterning process of developing muscle fibers proved to be the dihydropyridine receptor (DHPR), the L-type calcium channel in muscle. [87] This transmembrane protein acts as a voltage sensor. On depolarization of the sarcolemma, it activates the associated type 1 ryanodine receptor, leading to a release of calcium from the sarcoplasmatic reticulum, which then triggers muscle contraction. Chen *et al*. showed that in knockout mice lacking the β1 subunit of the L-type calcium channel DHPR (Cacnb1-/-), but also in double knockout mice lacking Cacnb1 and HB9, the pre-patterning of AChR clusters was lost. Nonetheless, synapses were established, but myofibers from DHPR deficient mice contained multiple endplates whereas the majority of control myofibers contained only a single endplate.

In summary, MuSK is involved in calcium-dependent aneural prepatterning of AChR clusters and in neural AChR cluster formation in the mature endplate. It is not clear if these aneural clusters are essential for NMJ formation, but they may serve as target for motor axons toward the centre of the muscle fiber. Neural agrin then binds through LRP4 to MuSK, causing autophosphorylation of the juxtamembrane domain and the active loop of the kinase. MuSK is thus switched to an active state which is enhanced by the binding of Dok7, creating a tetramer of two MuSK monomers and one Dok7 dimer. The β subunit of AChR is phosphorylated, enhancing its binding to the rapsyn scaffold protein. Activated MuSK is internalised and its active state is maintained by intracellular kinases, stabilising the NMJ independently of the presence of agrin. The essential role of most of these proteins in human neuromuscular transmission is further substantiated by the description of several congenital myasthenic syndromes involving mutations in MuSK, rapsyn, Dok7 or agrin. [88-91]

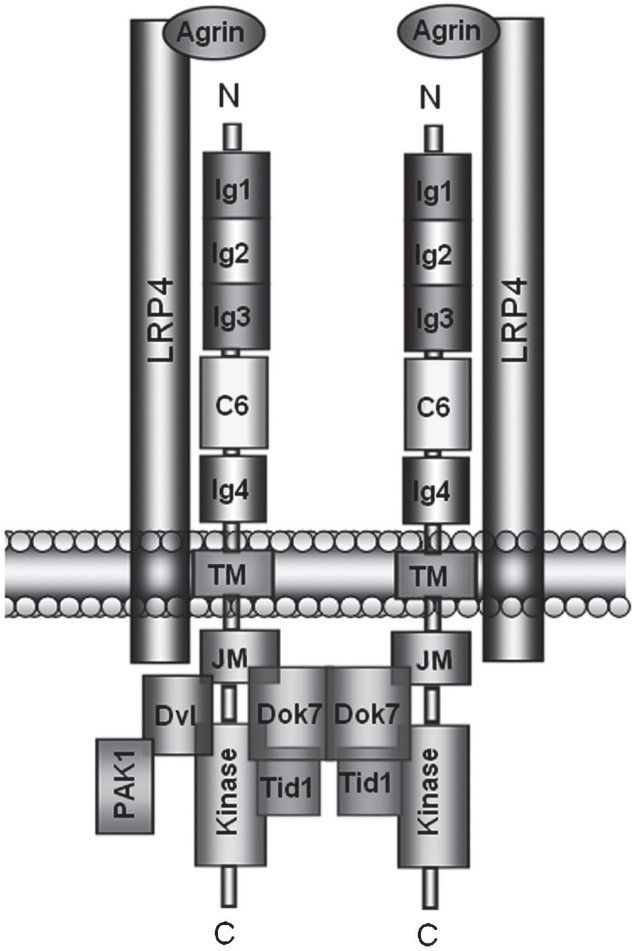

Figure 1.1 The assembled dimeric MuSK signalling complex
The extracellular domain contains 4 immunoglobulin-like domains and a cystein rich domain and interacts with the agrin binding receptor LRP4. Reprinted from the International Journal of Biochemistry and Cell Biology, Volume 43, Ghazanfari *et al*., Muscle specific kinase: organiser of synaptic membrane domains, Page 296, Copyright 2010, with permission from Elsevier Ltd.

Aims of the thesis

The aim of the studies in this thesis was to investigate the pathogenesis and the underlying immunological defect of MuSK MG. To reach this goal a detailed characterisation of the clinical course and features of patients with MuSK MG, and a study of the antibody response to the MuSK protein were first performed.
The discovery of anti-MuSK antibodies in a subset of patients classified until then as having "seronegative" MG (SNMG) raised the question whether these autoantibodies defined a new and distinct clinical disorder different from AChR MG. To investigate this, we first studied the epidemiology of MG subtypes in the Netherlands on a regional level using a well-defined area in the province of Zuid-Holland with a long-lasting and complete MG registry (Chapter 2). This enabled us to calculate population based data on the prevalence and incidence of the different subtypes of generalised MG.
We then undertook a nationwide study of patients with acquired generalised MG in the absence of AChR antibodies. This led to the identification of a relatively large cohort of SNMG and MuSK MG patients. Next, we described the clinical characteristics of MuSK MG patients and compared them to a cohort of AChR MG patients matched for sex and age at onset, and to patients with SNMG (Chapter 3). The immunopathological mechanism of MuSK MG was studied in more detail by investigating the association between MuSK MG and HLA haplotypes, described in Chapter 4. Longitudinal MuSK MG sera were collected to correlate antigen-specific IgG subclasses to clinical disease severity, thus substantiating their role in the immunopathogenesis (Chapter 5). In addition we searched for new antigens in AChR Ab-/MuSK Ab- patients. We hypothesised the existence of antibodies to the postsynaptic ErbB receptor, because ErbB is also a transmembrane protein accessible to an immunoresponse and involved in the development of the NMJ (Chapter 6).
Two case studies highlight the pivotal immunological role of IgG4 autoantibodies to MuSK (Chapter 7) and the electrophysiological characteristics of the neuromuscular transmission defect in MuSK MG (Chapter 8). Finally, overall results are summarised and discussed (Chapter 9).

II

Epidemiology of myasthenia gravis with anti-muscle-specific kinase antibodies in the Netherlands

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Abstract

The epidemiology of myasthenia gravis subtypes and the frequency of antibodies against muscle-specific kinase (MuSK) were studied in patients with generalised myasthenia gravis without anti-acetylcholine receptor antibodies who had an onset of symptoms between 1990 and 2004 in a well-defined region in the Netherlands. The nationwide prevalence and incidence myasthenia gravis with anti-MuSK antibodies were also studied. MuSK antibodies were found in 22% of patients with generalised myasthenia gravis without anti-acetylcholine receptor antibodies. Nationwide, 35 patients with MuSK myasthenia gravis were identified, yielding a prevalence of 1.9 per million (95% confidence interval (CI) 1.22 to 2.59) and an annual incidence of 0.10 per million person-years (95% CI 0.06 to 0.14).

Introduction

In North American and European cohorts, 38-47% of patients with generalised myasthenia gravis without anti-acetylcholine receptor antibodies (AChR Ab- MG) have antibodies to muscle-specific kinase (MuSK). [56,92,93] By contrast, this proportion is only 4% in Taiwan, and myasthenia gravis with anti-muscle-specific antibodies (MuSK Ab+ MG) seems to be absent in Norway, suggesting large regional differences. [94,95] We studied the epidemiology of myasthenia gravis subtypes, the proportion of MuSK Ab+ MG in patients with generalised AChR Ab- MG in a well-defined region in the Netherlands, and the nationwide prevalence and incidence of MuSK Ab+ MG.

Methods

Patients

Patients diagnosed with any form of myasthenia gravis in the densely populated northern part of the province of Zuid-Holland have been followed up by our centre since January 1 1990 as described previously. [96] All patients with myasthenia gravis, with an onset of symptoms up to January 1 2004 while living in this region were included in the regional study. In addition, all eight university medical centres and five larger general hospitals included patients with generalised AChR Ab- MG in the nationwide study up to January 1 2006 by listing patients under current treatment, searching computerised diagnosis registrations, and the use of patients identified in an earlier study. [31]

Inclusion criteria for AChR Ab- MG

The diagnosis was based on clinically confirmed fluctuating weakness of voluntary muscles acquired after the age of 2 years and the absence of anti-acetylcholine receptor (AChR) antibodies. Ocular myasthenia gravis was diagnosed when only diplopia or ptosis had been present throughout the course of the disease. The presence of mild weakness of lid closure was allowed for the diagnosis. Generalised myasthenia gravis was defined as the involvement of muscles other than external eye muscles, the levator palpebrae or the orbicularis oculi. The diagnosis was considered to be confirmed electrophysiologically if a decrement of the compound muscle action potential of more than 10% had been found during repetitive nerve stimulation or if single-fiber electromyography (EMG) had shown an increased jitter or blocking.

Data collection

The month in which the first MG symptoms had occurred was noted according to the patient's chart. Patients with AChR Ab- MG were asked for informed consent by their attending neurologist. Serum was tested for the presence of anti-MuSK, anti-AChR and anti-voltage-gated calcium channel antibodies using standardised immunoprecipitation assays (RSR Ltd., Pentwyn, Cardiff, UK) and patients were re-examined by EHN for confirmation of clinical criteria and the time of onset. Population figures were provided by Statistics Netherlands.

Statistics

Incidences were computed using the number of patients with the onset of symptoms between January 1 1990 and January 1 2004 and the total observed person-years. Prevalences were calculated on January 1 2004. Poisson distribution was used for 95% confidence intervals (CI).

Results

Regional patients

We identified 288 patients in whom myasthenia gravis had been considered. After reviewing their charts, 35 were excluded because of a revised diagnosis (n=30), congenital myasthenia gravis (n=2) or lack of sufficient data to confirm the diagnosis (n=3). Of the remaining 253 patients with clinical myasthenia gravis, 189 tested AChR Ab+. On January 1 2004, 160 patients were alive and 111 had an onset between 1990 and 2004. Information on the antibody status of eight patients was not available, leaving 56 patients with AChR Ab- MG. In this group, 30 had ocular myasthenia gravis (45% of all patients with ocular myasthenia gravis), in whom no anti-MuSK antibodies were found. In 26 patients with generalised AChR Ab- MG (14.5% of all patients with generalised myasthenia gravis), diagnosis had been confirmed electrophysiologically in 16 and by a positive response to acetylcholinesterase inhibitors in 8. In the other two patients the diagnosis had been based on clinical symptoms. Three patients died before the onset of the study. Among the remaining 23 patients, anti-MuSK antibodies were present in 5 (22%, 3 women and 2 men). All five were alive on January 1 2004 and four had an onset between 1990 and 2004. A flow chart of this classification of regional patients is presented in Figure 2.1.

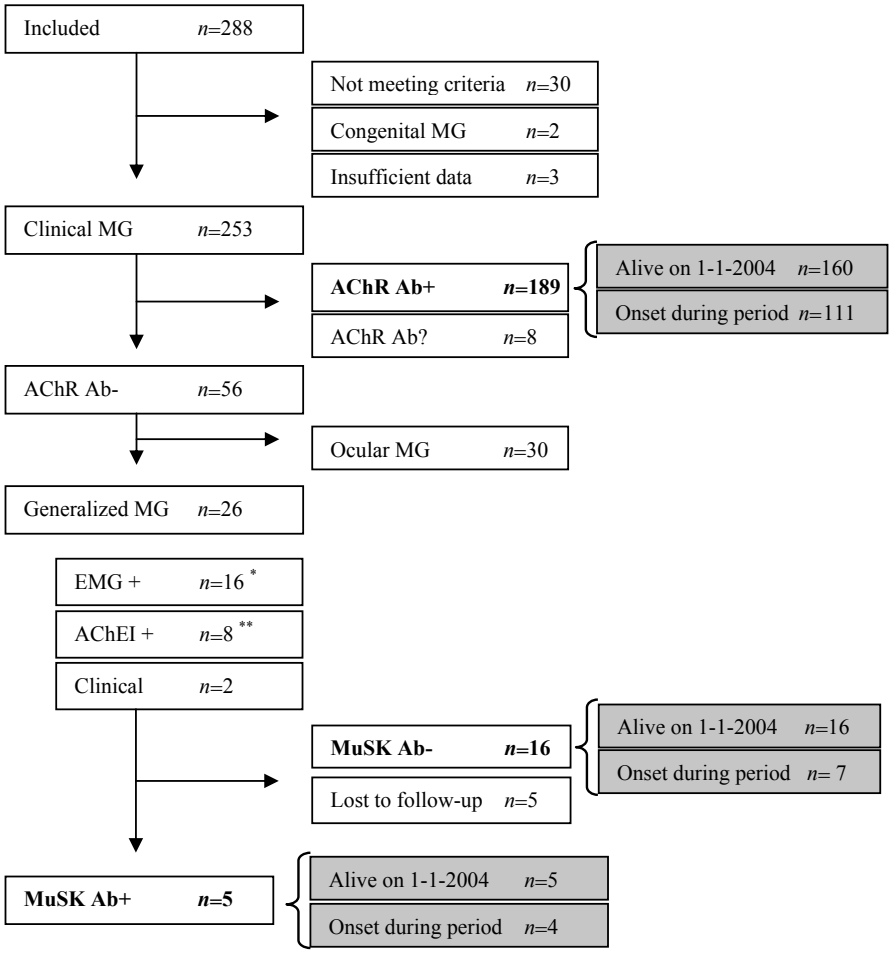

Figure 2.1 Regional patients classified according to MG subtypes
The diagnosis MG was confirmed by EMG (*) or by a positive response to an acetylcholinesterase inhibitor (**).

During this period, the regional population increased from 1 641 227 to 1 778 564, yielding a total of 23 926 703.5 observed person-years. Table 2.1 shows the prevalences on January 1 2004 and the average annual incidence of myasthenia gravis subtypes.

Table 2.1 Prevalence and incidence of myasthenia gravis subtypes

	Prevalence per million inhabitants (n)	95% CI	Incidence per million person-years (n)	95% CI
Regional MuSK Ab+ MG	2.8*	0.30 - 5.33	0.17*	0.00 - 0.33
Nationwide MuSK Ab+ MG	1.9*	1.22 - 2.59	0.10*	0.06 - 0.14
AChR Ab+ MG	90.0	75.8 - 104.2	4.64	3.76 - 5.52
Generalised AChR Ab-/MuSK Ab- MG	10.1	5.3 - 14.9	0.38	0.13 - 0.63

AChR Ab+ MG, myasthenia gravis with anti-acetylcholine receptor antibodies; AChR Ab- MG, myasthenia gravis without acetylcholine receptor antibodies; MuSK Ab- MG, myasthenia gravis without anti-muscle-specific kinase antibodies.
* Differences between regional and nationwide data are not statistically significant

Nationwide patients

We identified 130 patients diagnosed with generalised AChR Ab- MG. After clinical re-examination, 21 were excluded because of low anti-AChR titres in newly acquired serum (n=14) or not meeting clinical criteria (n=7). Anti-voltage-gated calcium channel antibodies were found in one patient. MuSK antibodies could not be determined in 11 patients because of a lack of informed consent (n=4), loss to follow-up (n=3) or death (n=4). Among the remaining 97 patients anti-MuSK antibodies were found in 35 (36%), of whom two died before the onset of the study. Thirty three patients were clinically re-examined. There was a female predominance (27 *vs.* 8). Median age at onset was 30.5 (range 2.0-74.6) years. We identified 30 white patients, two Creoles, one Persian, one Iraqi and one of Balkan origin. Median time from the onset of symptoms to a diagnosis of myasthenia gravis (not MuSK Ab+ MG), was 9 months (range 1 month-33 years). The neuromuscular transmission defect had been confirmed by EMG in 31 patients although no single-fiber EMG had been performed in the other four. Three of these four had shown a positive response to intravenous acetylcholinesterase inhibitors.

On January 1 2004, 31 patients were alive, and 22 had an onset between 1990 and 2004. The annual incidence fluctuated between 0 and 4 patients per year without a trend to increase. From January 1 1990 to January 1 2004, the population increased from 14 892 574 to 16 258 032 inhabitants. Nationwide prevalence and incidence rate of MuSK Ab+ MG are shown in Table 2.1.

Discussion

MuSK Ab+ MG is found only in a minority of patients with generalised AChR Ab- MG in the Netherlands. The difference between the regional and non-regional proportion of MuSK Ab+ MG is not statistically significant (odds ratio 0.41, 95% CI 0.14 to 1.22). The lower regional proportion of 22% seems a more accurate estimate coming from a population based registration of all myasthenia gravis subtypes. Regional prevalence and incidence of AChR Ab+ MG are comparable to those found in other European countries, varying between 77 and 105 per million and between 3.8 and 7.4 per million per year. [97] The proportions of AChR Ab- patients in the groups with ocular and generalised myasthenia gravis are comparable to historical data indicating that no large referral bias towards AChR Ab- MG occurred. Nationwide, not all patients with generalised AChR Ab-/MuSK Ab- MG who have often only mild generalised weakness, [56] may have been referred to one of the tertiary centres involved, increasing the proportion of patients with MuSK Ab+ MG. The higher proportion of 36% MuSK Ab+ MG nationwide is comparable to 38% in North American, 41% in British and 47% in Italian cohorts, and selection or referral bias may well cause this variation. By contrast, the low prevalence of MuSK Ab+ MG in Taiwanese and Danish patients (F. Somnier, oral communication) and its absence in Norway also suggest a genetic or environmental susceptibility, which was previously proposed in other autoimmune diseases. T helper cell 1-mediated diseases such as type-1 diabetes and multiple sclerosis have increased prevalences at higher latitudes, whereas pemphigus vulgaris, thought to be T helper cell 2 mediated, has lower prevalences at higher latitudes. [98] MuSK Ab+ MG is comparable to pemphigus vulgaris with regard to a predominance of IgG4 in active disease and an association with the HLA-DR14-DQ5 haplotype. [93,99] Epidemiologic data from other countries may be helpful to confirm regional differences in the prevalence of MuSK Ab+ MG and to identify possible aetiological mechanisms.

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III

Clinical comparison of myasthenia gravis subtypes reveals predominant limb-girdle and extraocular muscle weakness in seronegative patients

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Submitted

Abstract

The diagnosis of seronegative myasthenia gravis (SNMG) depends on the absence of antibodies to known antigens in the neuromuscular junction, and therefore on the sensitivity of the assays that have been used. Differences in clinical course between MG subtypes have been described, but studies often lack a standardised approach or a direct comparison of groups. In this nationwide study, we compared MuSK MG patients to AChR MG controls matched for sex and age of onset and to SNMG patients. Symptoms were investigated using standardised history taking and cross-sectional examination and analysed with survival statistics. New serum was acquired and tested for antibodies using radioimmunoassays. Forty-one SNMG patients, 25 MuSK MG and 50 AChR MG controls were included. During the course of the study, cell-based assays became available to detect antibodies to AChR or MuSK using immunofluoresence in human embryonic kidney cells with expressed recombinant AChR subunits and rapsyn, or over-expression of recombinant MuSK. These assays identified 10 patients with antibodies to overexpressed MuSK and another 10 with antibodies to clustered AChR. The phenotype of the remaining 21 SNMG patients was characterised by mild proximal limb-girdle weakness with prominent extraocular involvement. Apart from more proximal leg weakness in SNMG, symptoms in the first month did not discriminate between the subtypes. Analysing subsequent occurrence of symptoms, hazard ratios for dysarthria, dysphagia, neck weakness, and dyspnoea were significantly increased in MuSK MG compared to SNMG. Among bulbar symptoms in MuSK MG patients, chewing was relatively spared which seems consistent with MRI studies. The phenotype of MuSK MG patients identified by the cell-based assay was milder, but comparable, to that of the original MuSK MG cohort identified by radioimmunoassay. Cell-based assays are useful to define a more homogenous cohort of SNMG patients that can help in the search for new antigens in the neuromuscular junction.

Introduction

Fluctuating weakness of voluntary muscles is the clinical hallmark of myasthenic syndromes. In acquired autoimmune myasthenia gravis (MG), weakness is caused by antibodies directed to antigens in the neuromuscular junction. The antigen most frequently involved is the postsynaptic acetylcholine receptor (AChR). Autoantibodies to muscle-specific kinase (MuSK) in patients with AChR antibody negative MG were first described in 2001. [54] Recently, the low-density lipoprotein receptor-related protein 4 has been postulated as another antigen in AChR antibody negative MG, although some of these patients also have antibodies to MuSK and their pathogenicity remains to be proven. [100-102] A diagnosis of seronegative MG (SNMG) is based on clinical symptoms and depends by definition on the absence of antibodies and therefore on the sensitivity of the assays used to detect them. Ideally, the neuromuscular transmission defect should also be proven by electrophysiological studies. However, the sensitivity of these tests depends on the level of clinical involvement of the muscles under study. The tests are also technically challenging, often uncomfortable to patients, and therefore have not always been performed to the fullest extent. Antibodies to AChR or MuSK are demonstrated using radioimmunoassays (RIA). Modifications of the AChR assay first described in 1976 increased its sensitivity and limited the proportion of generalised SNMG patients. [42] In 2008, a cell-based assay was described using transfected AChR molecules clustered with rapsyn cotransfected in human embryonic kidney cells, defining a further 60% of SNMG patients as antibody positive. [43] In the present study, we introduce a similar approach to detect antibodies to MuSK in AChR antibody negative MG.
MG is a whimsical disease with significant variation in the distribution and severity of weakness between patients and between different muscles of the same patient. MuSK MG seems clinically distinguishable from AChR MG and SNMG because of predominantly bulbar weakness, affecting speech, swallowing and facial muscles in most patients, [56,92,103-110] although weakness of neck extensors can also be found. [92,105,107,111,112] Respiratory crises occur in approximately one quarter to one third of the patients. [56,92,104-107,110] Presenting symptoms are mainly ocular according to some. [56,108] However, an onset with (oculo)bulbar symptoms, weakness of neck extension, respiratory insufficiency or generalised weakness has also been reported. [92,104,111] After several years, the fluctuating character of the weakness seems to become less manifest in some of the patients, which is accompanied by atrophy of facial and tongue muscles. [56,103,104,107,110] Acetylcholinesterase inhibitors often do not improve weakness, [56,105,109,111] and can even lead to muscular fasciculations. [56,110]
SNMG emerges as a more heterogeneous disease. Weakness seems less severe in the majority of patients with predominant involvement of limb muscles and external eye muscles. However, bulbar, neck and respiratory muscles can be frequently affected as well. [56,105,111] Others claim that SNMG patients are more severely affected than AChR MG patients with respiratory

crises in nearly 20%. [106] Until now, none of the clinical studies applied a standardised protocol to assess the distribution of initial, presenting and most severe weakness or the course of symptoms in time. It is often not clear whether information is based on review of charts or standardised cross-sectional examination. The MGFA or Osserman's classifications often used offer only a limited description of clinical symptoms. [1,113] Three studies made a direct comparison between MuSK MG and AChR MG, but patients were included consecutively without some form of matching. [106,112,114] Only few studies have presented both cross-sectional data and information on the longitudinal course of the disease. [56,104] In all clinical studies, the definition of SNMG was based on conventional RIA.
Detailed information on the course of symptoms may help in the early diagnosis of this rare disorder. Knowledge of clinical patterns of weakness and response to therapy is also important for individual patients and their physicians, and for the design of future clinical trials. The newly developed cell based techniques to test for antibodies to the AChR or MuSK enable application of more rigorous criteria for SNMG. To study the symptomatology of MuSK MG and SNMG in detail and to investigate whether they are clinically different from AChR MG, we compared the course of symptoms, rate of progression, distribution of weakness and response to treatment in these three MG subtypes using to a standardised protocol.

Methods

Patients

Patients with generalised MG without AChR antibodies were included from a nationwide study as described previously. [115] In brief, charts from 130 patients with a diagnosis of acquired MG without AChR antibodies were screened in all eight University Medical Centers and five large General Hospitals in the Netherlands. Twenty-one were excluded because of low anti-AChR titres in newly acquired serum (n=14) or not meeting clinical criteria (n=7). Voltage gated calcium channel antibodies were found in one patient. MuSK antibodies were present in 35 patients, but could not be determined in 11 patients, either because of a lack of informed consent (n=4), loss to follow-up (n=3) or death (n=4). This yielded a total of 62 eligible SNMG patients.
The clinical course of the disease from the first 25 consecutively included patients with MuSK MG was compared to that of 50 matched control patients with generalised non-thymoma AChR MG. These controls were selected from our database containing a complete registration of all MG patients in the northern part of the province of Zuid-Holland since 1990. MuSK MG patients and controls were matched in a 1 to 2 ratio for sex and the decade in which the first symptoms had occurred using the random sample function from

SPSS 12.0.1 statistical software (SPSS Inc, Chicago, Illinois, USA). Patients were asked for informed consent by their attending physician. The medical ethical committees of all centres involved approved of the study.
All patients were re-examined by a single investigator (EHN) using a standardised history taking and physical examination. Using information from this history and the patients' chart, the month was noted in which various symptoms had occurred for the first time. The symptoms and signs under investigation were diplopia, ptosis, impaired facial expression, articulation, swallowing, chewing, and tongue movements, weakness of the neck (flexion or extension), dyspnoea, invasive ventilatory support, and proximal and distal muscles in arms and legs. Patients were excluded from clinical analysis if they had insufficient recollection of the course of their disease during these first years and if letters and notes in their chart did not provide this information. Patients were also excluded who had coexisting diseases with symptoms not distinguishable from MG symptoms.

Definition of MG subgroups
The diagnosis of SNMG was primarily based on clinically confirmed fluctuating weakness of voluntary muscles other than external eye muscles, the levator palpebrae or the orbicularis oculi, acquired after the age of 2 years, and the repeated absence of AChR antibodies in at least two assays. The diagnosis was considered to be confirmed electrophysiologically if a decrement of the compound muscle action potential of >10% had been found during repetitive nerve stimulation (RNS) or if single-fiber electromyography (SFEMG) had shown an increased jitter or blocking.
Serum from all patients was retested for antibodies to the AChR, MuSK, voltage-gated calcium channels (VGCC) using radioimmunoassays (RSR Ltd., Cardiff, UK) and for creatine kinase (CK). In all SNMG patients, DNA was tested for the rapsyn N88K mutation. The sera were then also retested to identify antibodies to clustered AChR or overexpressed MuSK. For this, recombinant AChR subunits with rapsyn or recombinant MuSK full length were expressed in transfected human embryonic kidney (HEK) cells growing on coverslips. To identify the expressed AChR clusters or the MuSK on the cell surface, both rapsyn and MuSK were tagged with enhanced green fluorescent protein (EGFP). Serum sample was applied at the dilution of 1:20. Cells were fixed and human antibodies were visualised by anti-human Alexa Fluor-labelled secondary antibody. Coverslips were examined on fluorescence microscope by two independent examiners using a semi-quantitative scoring system (0 to 4). The average score was used to calculate the antibody levels with expressed recombinant AChR subunits and rapsyn, or recombinant MuSK.

Statistical analysis

Kaplan-Meier curves

Inverted Kaplan-Meier curves were used to plot the cumulative incidence of symptoms during the 5 years following the first symptom. Statistical comparisons between the three groups were made using Pearson Chi-Square followed by Fisher's exact tests for the proportion of patients that had a specific symptom already in the first month (t=0 to t=1), and using the log-rank test for the risk of developing each symptom between t=1 and t=60 months, *i.e.* during the first 5 years of follow-up. P-values were considered statistically significant below 0.05 using the Bonferroni-Holm correction for multiple comparisons. A Cox proportional hazard model was then used to calculate the hazard ratios for each symptom to occur in the patients not presenting with that symptom. This model was also used to evaluate the matching for sex and age at onset between MuSK MG patients and AChR MG controls and to what extent these variables had influenced the comparison between MuSK MG and SNMG. All statistical analyses were performed using the SPSS 17.0 statistical software (SPSS Inc, Chicago, Illinois, USA).

Results

Patients

MuSK MG patients

In the 25 MuSK MG patients a female predominance was found (76%). Age at onset varied substantially between 6 and 74 years which was comparable to the range of the AChR MG controls although the mean age at onset was slightly higher in the MuSK MG group (Table 3.1).

SNMG patients

Of the 62 patients with SNMG described in the previous epidemiological study, 7 were lost to follow-up, no informed consent was obtained from 6, 1 patient had died and 1 16-year-old girl had become AChR antibody positive three years after the onset of symptoms (Figure 3.1). The remaining 47 were clinically re-examined. After the examination, 6 patients were excluded from the analysis because symptoms were incompatible with generalised MG (n=2), homozygous N88K rapsyn mutation (n=1), documented fluctuating weakness ever since the first day of life compatible with a congenital myasthenic syndrome (n=1), comorbidity in the form of severe Cushing's disease before the onset of MG symptoms (n=1), and insufficient recollection and documentation (n=1).

Cell based assays

In the remaining 41 patients, characterisation of autoantibodies using the cell based assays yielded a further 10 patients with antibodies to the AChR, further referred to as 'clustered AChR' and 10 with autoantibodies to MuSK, further referred to as 'overexpressed MuSK'. These results left 21 patients with generalised SNMG (Figure 3.1). For the 10 patients with antibodies to overexpressed MuSK, 2 patients were scored as 3, 7 patients as 2, and 1 patient as 1 using the semi-quantitative system and two independent examiners. For the 10 with antibodies to clustered AChR, 5 patients were scored as 1, one patient as 3, and 4 between 1.5 and 2.5. Examples of the scores are shown in Figure 3.2.

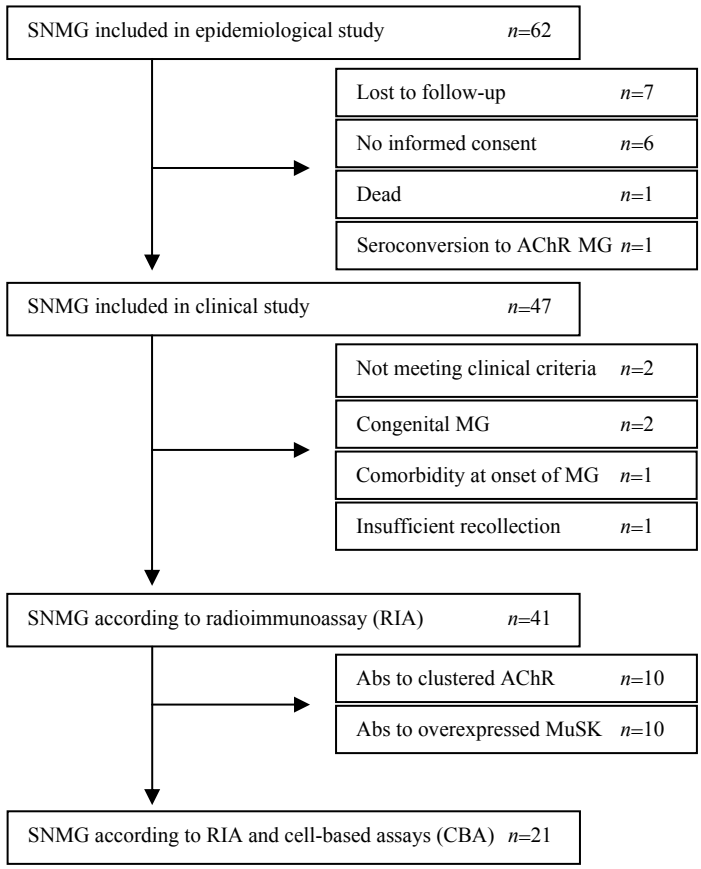

Figure 3.1 Inclusion and selection of SNMG patients
New serum and DNA was obtained from all patients included in the clinical study at the time of the cross-sectional examination. This serum was tested for antibodies to AChR and MuSK using radioimmunoassay (RIA). DNA was tested for the N88K rapsyn mutation. During the course of the study, cell-based assays became available and were used to test for antibodies to clustered AChR or overexpressed MuSK.

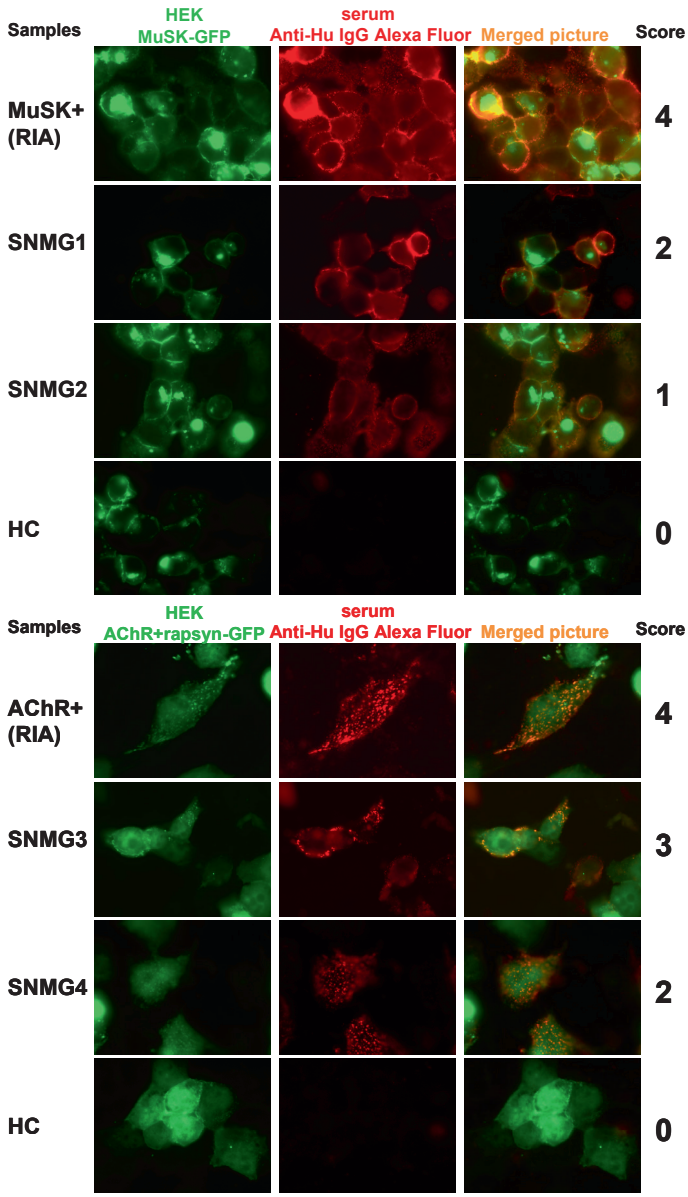

Figure 3.2 Examples of positive samples for antibodies to MuSK or AChR in the cell-based assays
Left handed column shows transfected HEK cells with either overexpressed MuSK (4 rows at the top), or clustered AChR and rapsyn (4 rows at the bottom) tagged with enhanced green fluorescent protein (GFP). The middle column shows bound IgG visualised by anti-human Alexa Fluor-labelled secondary antibody from 4 SNMG patients (defined by RIA in the original cohort). The right handed column shows the merged pictures. Coverslips were examined by two independent examiners using a semi-quantitative scoring system (0 to 4). The first row of each section shows an example of patients who tested positive for MuSK (top) or AChR (bottom) in the RIA. HC = Healthy Control.

Clinical characteristics

Demographic and clinical characteristics of the 25 MuSK MG patients, 50 AChR MG controls and 21 patients with generalised SNMG are shown in Table 3.1. There was a female predominance in MuSK MG and SNMG. Age at onset and the number of patients with an onset in childhood were also similar. The indicated delay in diagnosis refers to the diagnosis of an acquired myasthenic syndrome, because in many patients the test for MuSK antibodies was not available at that time. The same applies for some of the older AChR MG patients with an onset before 1978. The wide range of the delay in the MuSK MG group is mainly due to one patient who had very prolonged static bulbar symptoms and atrophy of tongue and facial muscles for more than 30 years, following a relatively brief period of fluctuating ocular symptoms. One AChR patient had been treated with low dose prednisone for a suspected inflammatory myopathy between 1972 and 1981 before the diagnosis of MG was made. When excluding these two cases, the maximum delay was 5.9 years in the MuSK MG group and 4 years in the AChR MG group. At maximum disease severity, the MuSK MG patients were scored more often as predominantly bulbar (indicated by MGFA class IIb, IIIb or IVb), and twenty-eight percent required invasive ventilatory support at that time. By contrast, only one SNMG patient had required ventilatory support for one week after which symptoms resolved upon treatment with prednisone and azathioprine. Interestingly, this occurred at the age of 72 years, 40 years after the onset of the generalised symptoms. The majority of patients in all three groups had been treated with immunomodulating therapy at some point during the course of the disease. The severe involvement of bulbar symptoms in MuSK MG was also substantiated by a more frequent occurrence of severe weight loss compared to AChR MG and SNMG. However, in four AChR MG patients a predominantly bulbar phenotype was present as well, requiring nasogastric tube feeding without the need for invasive ventilatory support.

Table 3.1 Demographical and clinical characteristics of three groups of MG patients

	MuSK	AChR	SNMG
No. of patients	25	50	21
Women – *n* (%)	19 (76)	38 (76)	12 (57)
Non Caucasian - *n* (%)	3 (12)	2 (4)	0 (0)
Age at onset - median (range) in yrs	31.0 (6.0-74.5)	27.9 (4.6-78.3)	39.3 (5.9-66.8)
Age at onset below 16 yrs - *n* (%)	4 (16)	11 (22)	4 (19)
Delay in diagnosis - median (range)	0.7 (1m-33.1y)	0.5 (20d-9.9y)	1.6 (2d-13.0y)
Follow-up - median (range) in yrs	9.1 (1.2-33.8)	12.9 (0.9-41.2)	10.3 (0.8-45.5)
Duration to maximum (median and range in years)	1.1 (0.4-32.5)	1.1 (0-19.0)	3.3 (0-40.5)
MGFA at onset - *n*			
- I	11	24	10
- II (b)	13 (8)	24 (9)	11 (2)
- III (b)	1 (1)	2 (1)	0 (0)
MGFA at maximum - *n*			
- II (b)	1 (1)	5 (3)	9 (1)
- III (b)	9 (8)	29 (5)	9 (3)
- IV (b)	8 (7)	11 (4)	2 (1)
- V (%)	7 (28)	5 (10)	1 (5)
Number of MGFA V episodes (Incidence per 100 patients per year)	11 (4.4)	15 (1.9)	1 (0.3)
Weight loss more than >10% *n* (%)	16 (64)	14 (28)	3 (14)
Tube feeding *n* (%) (While not on respiratory support)	1 (4)	4 (8)	0 (0)
Immunomodulation (%)	22 (88)	43 (86)	13 (62)
Thymectomy (%)	9 (36)	31 (62)	5 (24)
Thymic hyperplasia according to pathology report (%)	1 (11)	12 (39)	1 (5)
Interval to immunomodulation (median and range in yrs)	0.9 (0.4-6.9)	1.3 (0.2-10.6)	3.1 (0-40.5)

Diagnostic tests in SNMG

In the SNMG patients, the diagnosis was confirmed by abnormal repetitive nerve stimulation or single-fiber EMG in 15 (71%). In three patients with incomplete EMG studies, a positive response to IV acetylcholinesterase inhibitors (AChEI) was seen. In one patient with a positive AChEI response, RNS and SFEMG studies were normal. In the remaining 2 patients all three tests had been negative, and the diagnosis of acquired MG had been based on clinically confirmed fluctuating oculobulbar weakness responding to oral immunosuppressive therapy. Serum CK levels were normal for all patients included. Antibodies to VGCC were absent.

Evolution of symptoms

First symptoms

The first symptoms reported by the patients did not discriminate between MuSK MG, AChR MG, and SNMG. Isolated diplopia or ptosis was most frequently reported at the onset of the disease and occurred in 44% of the MuSK MG patients, in 50% of those with AChR MG

and in 43% of the SNMG group. In MuSK MG and AChR MG, the next frequently involved first symptoms were bulbar (20% in MuSK MG and 16% in AChR MG) and weakness of arms and legs (12% for MuSK MG versus 16% for AChR MG). In SNMG however, the next frequent first symptom was either simultaneous weakness in arms and legs (24%) or isolated weakness in the legs (19%). Two symptoms reported at onset only in MuSK MG were isolated neck weakness in two and severe dyspnoea in one.

Symptoms in the first month

For symptoms occurring within the first month, Pearson's Chi Square test showed a statistically significant difference between the three groups only for dyspnoea (p = 0.013) and proximal leg weakness (p = 0.029). Subsequent Fisher's exact tests showed that this was due to a more frequent occurrence of proximal leg weakness in 48% of the SNMG patients compared to 12% of the MuSK MG patients (p = 0.01), and of dyspnoea in 4 MuSK MG patients compared to none of the AChR MG patients (p = 0.01). In the first month, proximal weakness of the extremities was more frequent in AChR MG patients than in MuSK MG patients (28% *vs*. 8% for the arms and 34% *vs*. 12% for the legs), although this failed to reach statistical significance (p = 0.071 and 0.054). None of the other studied symptoms showed any statistically significant differences between the three MG subtypes during the first month.

Progression of symptoms after the first month

The cumulative incidences of 14 symptoms in the three MG groups during the 60 months after the onset of symptoms are represented by inverted Kaplan-Meier curves in Figure 3.3. The more frequent occurrence of symptoms in MuSK MG, as compared to AChR MG, reached statistical significance for diplopia, (p = 0.017), dysphagia (p = 0.003), neck weakness (p = 0.0002), dyspnoea (p = 0.0008), and intubation (p = 0.007). After two years, 92% of MuSK MG patients had experienced a period of diplopia, compared to 64% of AChR MG patients and 71% of SNMG patients (71%). After 5 years of follow-up, 28% of the MuSK MG patients had needed respiratory support, compared to only 6% of AChR MG patients and none of the SNMG patients. By contrast, the frequent occurrence of ptosis was similar in MuSK MG and AChR MG (78-80% during the first 2 years).

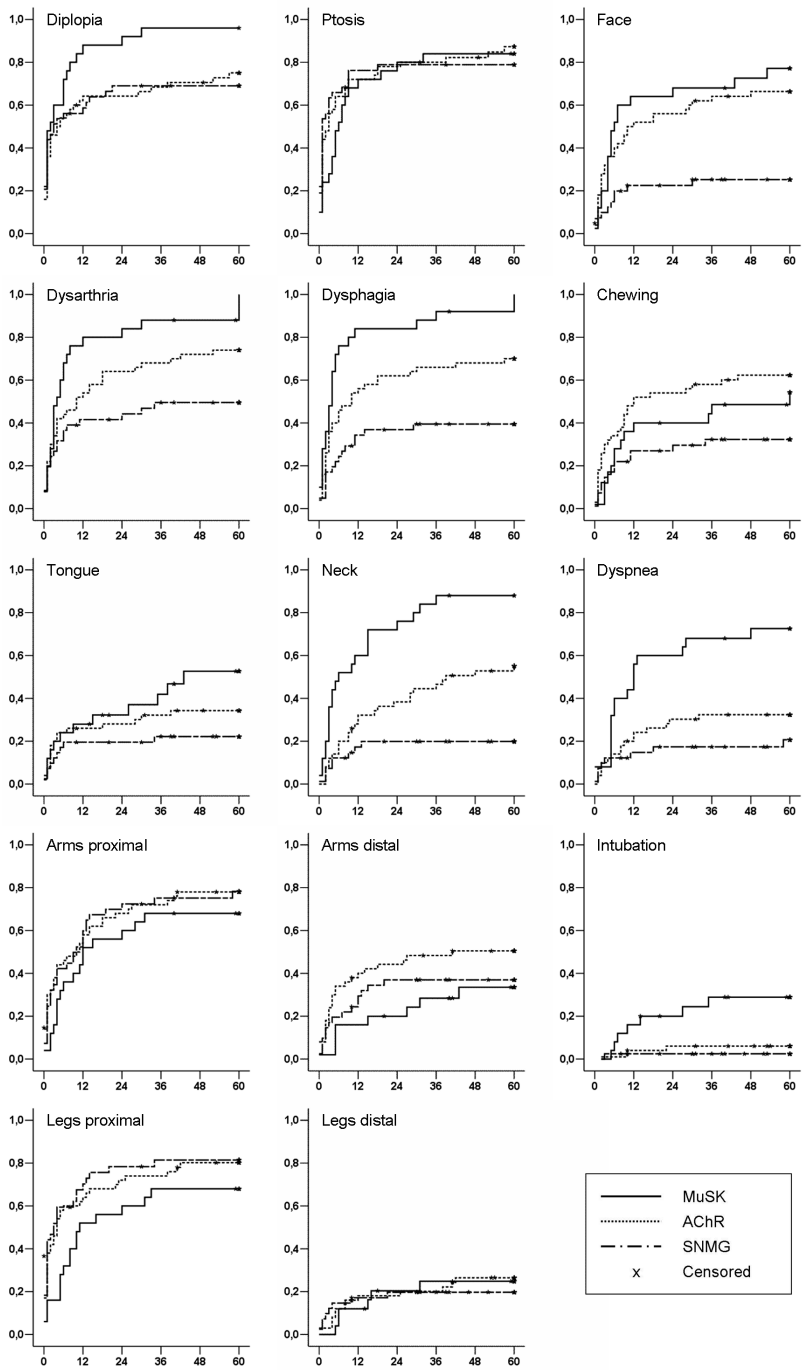

Figure 3.3 Inverted Kaplan-Meier plots indicating the cumulative incidences of 14 symptoms in the three MG groups during the 60 months after the onset of the first symptom

SNMG emerged as a disease with early and frequent occurrence of ptosis, followed by diplopia and proximal weakness in the arms and legs. The following symptoms in SNMG patients were significantly less frequent: facial weakness ($p = 0.001$), dysarthria ($p < 0.003$), dysphagia ($p < 0.001$), neck weakness ($p < 0.001$), dyspnoea ($p < 0.001$) and intubation ($p = 0.01$). Cox proportional hazard ratios for the 14 symptoms are given in Table 3.2. These ratios indicate the risk for a symptom to occur after the disease had presented itself in the first month with other symptoms. Upon addition of the matching factors age at onset and sex the hazard ratios in the Cox model for the comparison between MuSK MG and AChR MG remained similar (median proportion of hazard ratios 0.96, range 0.88 to 1.12). For the comparison between MuSK MG and SNMG, the median proportion of hazard ratios before and after correction for age at onset and sex as was 0.94 (range 0.65 to 1.16).

Table 3.2 Cox proportional hazard ratios for the 14 symptoms in patients with MuSK MG compared to AChR MG and SNMG patients

	MuSK MG compared to:			
	AChR MG		SNMG	
	Hazard ratio	*p*	Hazard ratio	*p*
Diplopia	2.2	0.031	2.3	0.075
Ptosis	1.0	0.918	0.8	0.466
Facial weakness	1.4	0.264	4.9	0.004
Dysarthria	1.9	0.027	**4.6**	**0.002** *
Dysphagia	2.0	0.017	**6.9**	**<0.001** *
Weakness of chewing	0.7	0.367	2.9	0.063
Weakness of tongue	1.5	0.341	3.4	0.063
Weakness of neck	**2.6**	**0.002** *	**9.7**	**<0.001** *
Dyspnoea	2.4	0.018	**9.7**	**0.003** *
Intubation	5.3	0.016	62.7	0.183
Arms proximal	1.0	0.888	0.9	0.859
Arms distal	0.6	0.287	5.8	0.1
Legs proximal	0.8	0.537	1.2	0.673
Legs distal	1.2	0.738	57.7	0.228

* *P*-value reaching statistical significance using the Bonferroni-Holms correction for 14 comparisons. Statistically significant results indicated in bold

In Figure 3.4, median time interval between onset and the first occurrence of 13 symptoms in three MG subtypes is shown. In SNMG patients, the median for ptosis and proximal limb weakness was reached earlier than in MuSK MG or AChR MG. The median for bulbar symptoms (facial expression, speech, swallowing, chewing and tongue movements) was not reached for SNMG, because during the follow-up, less than 50% of SNMG patients developed any of these symptoms.

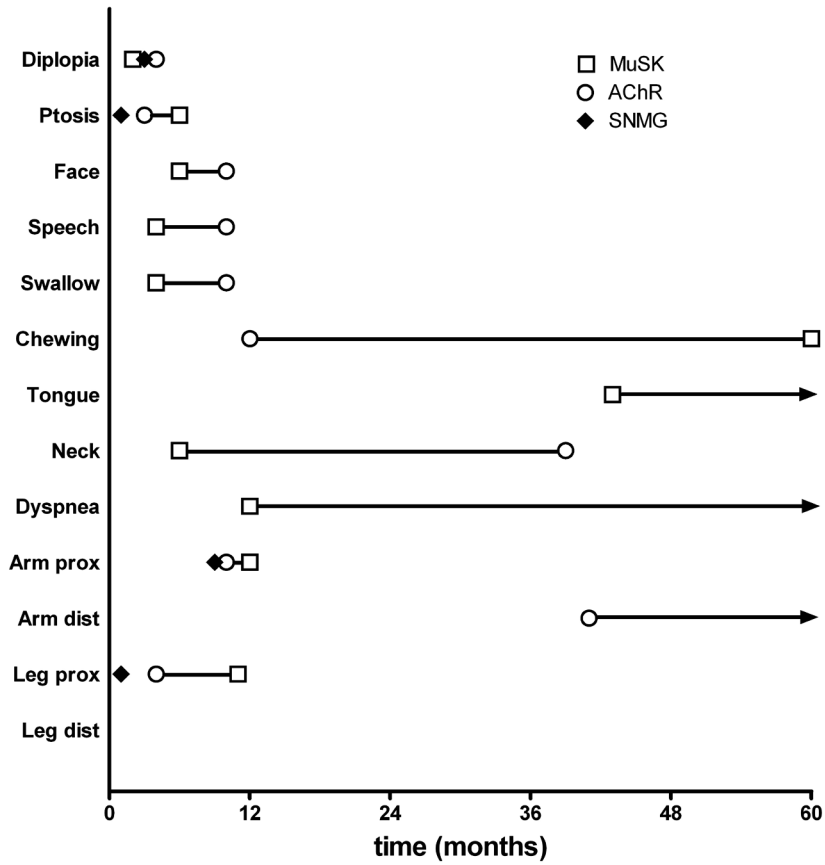

Figure 3.4 Median time interval between onset and first occurrence of 13 symptoms in MG subtypes
For the first occurrence of invasive ventilation and distal leg weakness, the median was not reached in any group.

Cross-sectional examination

MuSK MG (n=25)

Extraocular symptoms had often disappeared at cross-sectional examination. Although diplopia had been present somewhere during the course of the disease in all but one patient, diplopia was absent in 17 patients and severe in only 3. Ptosis was also absent in 17 and severe in 1 patient. Thirteen patients (52%) did not have any ocular symptoms.

The most frequently involved bulbar symptom was dysphagia, (severe in 3 and mild in 12) and dysarthria (severe in 5 and mild in 8). Sixteen patients had reported a period of body weight loss of more than 10% in the course of the disease. One patient had required tube feeding. Facial weakness was found in 14 patients of whom 9 had atrophied facial muscles. The tongue was weak in 12 patients and atrophied in 7. The jaw muscles were least involved and just mildly weak in 2 patients. Ten patients complained of dyspnoea, mostly related to exercise and increasing in supine position, compatible with diaphragmatic weakness. Bulbar symptoms were absent in only 4 patients of whom two were in clinical remission, one had mild diplopia and neck weakness, and one had weakness of the neck and arms.

Neck weakness was found in 68% of patients. Both isolated weakness of neck flexors (n=8) and neck extensors (n=1) was observed. Weakness of axial and limb muscles tested was symmetrical and within the MRC class 4 range except for 2 patients who could barely lift their head in prone position.

The arms were affected in 13 patients of whom weakness was exclusively proximal in 6 and only affecting distal muscles in 2. In the legs, only hip flexors and foot extensors were mildly affected in 7 patients. Knee extension and foot flexion were always within the MRC 5 range although 4 patients showed fatigability and were unable to perform 20 squads. Isolated foot extensor weakness was found in three.

At cross sectional examination, only two patients were in clinical remission, one in complete stable remission and one in pharmacological remission while on azathioprine. All patients but three had received immunosuppressive therapy (20 prednisone, 8 azathioprine, 7 plasma exchange, 4 thymectomy, 1 IVIG). In five patients, post intervention status was unchanged at cross-sectional examination. One patient depended entirely on regular plasma exchange using an arteriovenous shunt. Twenty-two patients had received pyridostigmine, leading to a clinical improvement of weakness in only 4, but to muscle twitching in 17 patients.

SNMG (n=21)

Weakness was in general much milder in the SNMG group. Extra ocular symptoms predominated in all but three patients. Ptosis was found in 14 and diplopia in 7. Bulbar muscles were affected in 5 patients only. Three had mild dysphagia and dysarthria. One showed severe facial weakness with atrophy. One complained of mild dyspnoea. No patient had required artificial ventilation throughout the course of the disease, except one who

suffered from pneumonia caused by rotavirus at the age of 72 and more than 40 years after onset of MG symptoms. Three patients had experienced more than 10% weight loss in the course of the disease.
Axial and limb muscles were all within the MRC grade 4 range. In the arms, proximal muscles were affected in 14 patients of whom 3 also had some distal weakness. In the legs proximal weakness was found in 12 of whom three had also distal weakness.
The milder course of the disease was also reflected by a less frequent use of immunosuppressive therapy compared to MuSK MG or AChR MG. Nine patients had never received oral immunosuppressive therapy of whom 2 had been thymectomized. Of the remaining 12 who received prednisone (n=7) or azathioprine (n=5), the clinical response to treatment was clear in 7 and somewhat questionable in 3. At cross-sectional examination, twelve patients (56%) had no immunosuppressants any more. Pyridostigmine had been prescribed in all but one patient and had been effective in 16 (80%). Only one female patient had reported muscle twitching.

Overexpressed MuSK (n=10)
In patients who tested negative for MuSK antibodies in the conventional RIA, but positive in the cell-based assay (overexpressed MuSK), there was a female predominance (8 *vs.* 2). Eight patients had shown a clinical course typical for MuSK MG with predominant bulbar weakness, mild proximal weakness and mild transient ocular symptoms. The other two had shown limb-girdle weakness affecting the legs more than the arms. In general, the course of the disease had been milder than that of the MuSK MG group with exception of one female patient who developed severe respiratory insufficiency within three months after onset. Six had never received immunosuppressive therapy. Apart from one female who adjusted meals for dysphagia, and another whose limb-girdle weakness limited her activities like housekeeping, no limitations in daily life activities were reported. At cross-sectional examination, only mild residual weakness was found in a minority of patients. None had ocular symptoms and four showed mild bulbar weakness. The tongue was atrophied in one. Three had mild weakness in the arms, and two in the proximal legs. Three were still treated with low dose oral immunosuppressive therapy. All had been treated with pyridostigmine. This was beneficial in 4, without any effect in 5 and caused muscle twitching in one.

Discussion

In this retrospective study we compared the course of clinical symptoms in MuSK MG in the first 5 years to that of AChR MG in two cohorts matched for sex and age at onset. This comparison was also made with a large cohort of SNMG patients identified by a nationwide

study. The SNMG cohort could be defined more strictly than in the studies published to date by excluding patients with antibodies to clustered AChR or overexpressed MuSK using cell-based assays. Antibodies to MuSK or AChR were found in 48% of the SNMG patients originally defined by RIA and were also mutually exclusive.

The three MG subtypes could be distinguished on a group level by studying fourteen areas of muscle weakness. MuSK MG emerged as a disease with predominant bulbar and axial weakness, affecting both neck flexors and extensors and leading to respiratory crises in one third of the patients. Almost all patients experienced periods of extraocular weakness early in the course of the disease whereas this was not a frequent symptom later on. Within the group of bulbar muscles, those involved in chewing were less affected than the ones used in articulation, swallowing or facial expression. The relative resistance to weakness of masseter muscles has also been described in an MRI study of MuSK MG. [116] Muscle atrophy and hypersensitivity to AChE inhibitors occurred frequently. Clinical characteristics of the overexpressed MuSK group were similar to that of the MuSK cohort defined by RIA. We also observed patients in this group who became seropositive in the MuSK RIA later in the course of the disease. This indicates that the cell-based assay is indeed able to detect antibodies that are clinically relevant to the diagnosis. By contrast, weakness in SNMG was generally of a mild proximal limb-girdle distribution with prominent extraocular involvement.

On the individual level however, fulminant and severe bulbar weakness were also seen in the AChR group and even in one SNMG patient. This emphasizes the whimsical nature of MG and makes it impossible in our view to clinically differentiate between the subtypes in individual patients, especially early in the course of the disease.

The rarity of MG makes it impossible to study large cohorts in a prospective way. The long follow-up we were able to describe has the possible disadvantage of recall bias. Patients often have a clearer memory of the onset of ocular symptoms compared to proximal limb-girdle weakness. The fact that patients and clinical data had to be included from a nationwide study of AChR antibody negative MG made it impossible to blind the examiner for the diagnosis. We tried to limit this potential bias by structuring the interviews and collecting registered medical information from hospital files made at the time by their treating physicians.

It remains questionable whether the selected SNMG patients represent a homogeneous entity. Patients with a congenital myasthenic syndrome rarely present at a later age, [45] We were able to identify one patient, homozygous for the rapsyn N88K mutation, who presented in his twenties, although careful history did reveal minor symptoms of weakness from early childhood. One copy of this mutation has been found in all late-onset patients described until now. [117] Although we did not sequence the Dok7 gene, a recent study of a large cohort of acquired SNMG patients did not find any copy of the common c.1124_1127dupTGCC mutation found in late-onset patients. [118] The response to immunosuppressive treatment in the majority does not support a genetic origin. Recently, autoantibodies to low-density

lipoprotein receptor-related protein 4 (LRP4) have been described in patients with SNMG. [100-102] LRP4 is the agrin receptor activating MuSK. As extracellular protein it is a candidate to trigger autoimmune responses. Pevzner *et al*. used full length human LRP4 cDNA to transfect HEK293 cells and showed immunoreactivity of SNMG sera from 19 out of 38 patients. [101] However, staining was also positive in one out of 11 MuSK MG patients, and SNMG was defined by conventional RIA only. The authors describe a high prevalence of bulbar weakness and female predominance similar to MuSK MG and state in the discussion that the LRP4 phenotype cannot be distinguished from AChR MG. Higuchi *et al*. reported LRP4 antibodies in 9 of 300 SNMG patients, but also in 3 of 28 MuSK MG patients, using precipitation assay in which the extracellular domain of LRP4 is conjugated to luciferase. [100] Here too, LRP4 positive patients showed severe bulbar or limb weakness and SNMG is defined by RIA only. It is not stated if only patients with generalised MG were included. In view of the high rate of patients with antibodies to clustered AChR or overexpressed MuSK in our study, it seems likely that such patients may also be part of the aforementioned studies. Therefore, it is not yet clear if these antibodies represent an isolated MG subtype and are indeed pathogenic. Although we were unable to detect autoantibodies to LRP4 in our SNMG cohort using cDNA from rat (cowork with Coldefy and Burden, unpublished), it cannot be ruled out that LRP4 MG is another MG subtype within our SNMG cohort.

In summary, the cell-based assays enabled us to refine the SNMG cohort by excluding nearly 50% of the patients who had antibodies to clustered AChR or overexpressed MuSK, showing more consistent limb-girdle weakness and ocular symptoms in the group of SNMG patients. Nonetheless, the pattern of weakness could vary significantly between individual patients, reflecting either the existence of multiple other antibodies to unknown antigens in the neuromuscular junction, late-onset congenital syndromes, or an unexplained intrinsic quality of myasthenia gravis in general.

IV

Strong association of MuSK-antibody positive myasthenia gravis and HLA-DR14-DQ5

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Abstract

The authors studied the HLA profile of 23 white Dutch patients with muscle-specific kinase antibody-positive myasthenia gravis (MuSK MG) and found an association with HLA-DR14-DQ5 (odds ratio 8.5; 95% CI 3.9-18.7; $p = 4.9 \times 10^{-5}$). Fifty-two percent of the patients carried the DR14 allele compared to 5% percent of the controls ($p = 1.0 \times 10^{-8}$). This association between MuSK MG and a relatively rare HLA haplotype differs from the previously described association of early-onset AChR MG with HLA-B8-DR3.

Introduction

In approximately 90% of patients with generalised weakness, myasthenia gravis (MG) is caused by autoantibodies directed against the nicotinic acetylcholinereceptor (AChR). In 30% to 40% of the patients with generalised MG without anti-AChR antibodies (AChR Ab- MG), antibodies against muscle-specific kinase (MuSK) can be found. MG with anti-MuSK autoantibodies (MuSK MG) comprises a clinical phenotype with marked involvement of oculobulbar, neck and diaphragmatic muscles. [56] Anti-MuSK antibodies are mainly of the immunoglobulin (Ig) G4 subclass, instead of IgG1 and IgG3 in AChR MG. [93]
The presence of other autoimmune diseases in patients with AChR MG and their first-degree relatives suggests a role for a genetic predisposition of which the HLA genotype is the most well known. Early-onset AChR MG without thymoma is strongly associated with the HLA-A1-B8-DR3 haplotype in white Europeans. [20-22] DR16 and DR9 are also linked to AChR MG in white French people. In this cohort, an association with polymorphisms in the gene encoding the α-subunit of the AChR as well as a decreased frequency of the DR7 allele has been found. DR2 has been associated with late-onset AChR MG. We studied HLA polymorphisms in white Dutch patients with MuSK MG.

Methods

Between July 2003 and December 2004, patients known to have acquired generalised AChR Ab- MG from all eight University Medical Centres and five larger general hospitals in the Netherlands were included in a nationwide study of seronegative MG. Patients were selected using computerised diagnosis registrations and asked for informed consent by their attending neurologist. A single investigator (E.H.N.) re-examined all patients to assess present clinical symptoms. Course of the disease and evolution of clinical symptoms in time were evaluated retrospectively by taking a detailed history from each patient, the use of questionnaires and review of patients' charts. Weakness during the onset of clinical symptoms and maximum disease severity was graded according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification. The presence of other autoimmune diseases in the patients and their first-degree relatives was evaluated during the interview. Serum from all patients was obtained and retested for the presence of anti-AChR, anti-MuSK and anti-voltage-gated calcium channel (VGCC) antibodies in Leiden using commercial assays (RSR Ltd., UK) and in Oxford. DNA was isolated from peripheral white blood cells. HLA Class I and II typing was performed with the use of PCR-amplified fragments and biotin-labelled oligonucleotides as previously described. [119] DNA typed DRB1* and DQB1* alleles were translated throughout to their serologic equivalents according to the World Health Organization nomenclature

committee. Frequencies of HLA alleles were compared to those of a previously published group of 2,440 healthy white Dutch blood donors. [120] Distribution of HLA haplotypes was compared to that of 321 individuals from the same control group available for haplotype assignment. For statistical analysis the two-sided Fisher exact test was used. *p* values were corrected for multiple informative comparisons conform the Bonferroni method. Odds ratios (ORs) with 95% CIs were calculated according to the Woolf Haldane test. All patients gave written informed consent before their participation. The Medical Ethical Committees of all hospitals involved approved the study.

Results

Patient characteristics

Seventy-three patients with acquired generalised AChR Ab- MG were found in the Netherlands. Anti-MuSK antibodies were present in 26 of whom 23 white patients (18 women and 5 men) were included. None of these patients had anti-AChR antibodies in at least two assays (range 2 to 14) performed in different laboratories. No anti-VGCC antibodies were found. The mean age at onset was 36.4 years (range 6.0 to 74.5 years). The mean duration of follow up was 10.4 years (range 1.2 to 33.9). The mean time from onset of symptoms until the first period of maximum disease severity was 1.8 years (range 0.25 to 6.75), apart from one patient whose weakness was greatest 32 years after onset. Weakness at onset of symptoms was restricted to the extraocular muscles in 8 (MGFA Class I), predominantly bulbar in 10 (MGFA Class IIb or IIIb), mainly affecting the extremities in 2 (MGFA Class IIa) and mixed generalised in 3 (MGFA Class II). At maximum disease severity, however, bulbar and respiratory muscles were prominently involved in 15 patients (MGFA Class IIb, IIIb and IVb), with a further 7 requiring ventilatory support (MGFA Class V), leaving only 1 patient with severe mixed generalised weakness (MGFA Class IV).

HLA association

The absolute and relative distribution of HLA polymorphisms in patients and controls were calculated (Table 4.1). A highly significant association was found with alleles DR14 (52% *vs*. 5% in healthy controls) and DQ5 (78% *vs*. 35%). Nineteen of 23 patients carried either DR14, or DQ5, or both. The HLA-DR14-DQ5 haplotype was significantly increased among MuSK MG patients (p_c = 4.9 x 10^{-5}, Table 4.2). The frequency of the DQ6 allele was decreased in MuSK MG (22% *vs*. 50%). There was no significant association with the B8-DR3 ancestral haplotype or with HLA Class I alleles.

Table 4.1 Frequencies of HLA alleles in 23 patients with MuSK Ab+ MG.

HLA*	MuSK n *(%)*	Controls n *(%)*	OR	95% CI	*p*	p_c §
A1	4 (17.4)	747 (30.6)	0.5225	0.1868 - 1.4616	0.2539	0.9778
B8	3 (13.0)	554 (22.7)	0.5809	0.1862 - 1.8117	0.3275	0.9974
DR3	2 (8.7)	599 (25.0)	0.3484	0.0937 - 1.2952	0.0885	0.6712
DR1	9 (39.1)	473 (19.7)	2.6601	1.1666 - 6.0657	0.0320	0.3235
DR14	12 (52.2)	127 (5.4)	18.8448	8.2956 - 42.8093	8.5×10^{-10}	1.0×10^{-8}
DR16	2 (8.7)	43 (1.8)	6.1173	1.5960 - 23.4471	0.0701	0.5821
DQ5	18 (78.3)	300 (34.6)	6.3523	2.4271 - 16.6255	3.1×10^{-5}	1.5×10^{-4}
DQ6	5 (21.7)	453 (50.1)	0.2960	0.1132 - 0.7736	0.0098	0.0482

* To allow comparison with a large control population that was typed serologically, DNA typed DR and DQ alleles were translated to their serological equivalents.
§ Corrected *p* value according to the Bonferroni method for multiple comparisons.

Table 4.2 Distribution of HLA-DQ5 associated haplotypes in patients and controls

HLA	MuSK n *(%)*	Controls n *(%)**	OR	95% CI	p_c §
DR14-DQ5	11 (23.9)	23 (3.6)	8.5	3.9 - 18.7	4.9×10^{-5}
DR1-DQ5	9 (19.6)	61 (9.5)	2.4	1.1 - 5.1	n.s.
DR10-DQ5	1 (2.2)	7 (1.1)	2.8	0.5 - 16.5	n.s.
DR16-DQ5	2 (4.3)	7 (1.1)	4.8	1.1 - 20.6	n.s.

* This control cohort consisted of 321 individuals available for haplotype assignment.
§ Corrected *p* value for multiple comparisons.

Related autoimmune diseases

Five patients had another autoimmune disease: two had thyroid disease, one had vitiligo, and two had psoriasis. Four of these five patients carried the DR14-DQ5 haplotype. Eight patients had a first-degree relative with autoimmune disease: three with thyroid disease, one with vitiligo, three with rheumatoid arthritis, and one with psoriasis. The DR14-DQ5 haplotype was found in three of these eight, and three patients had DQ5 in combination with DR1. Only one patient, whose first-degree relative had vitiligo, carried the B8-DR3 ancestral haplotype.

Discussion

We found a highly significant association in MuSK MG with HLA-DR14 and DQ5. This association is different from the known association of the B8-DR3 haplotype with early-onset AChR MG. Our cohort of MG patients is representative of the MuSK MG patients described so far, because clinical signs and symptoms are similar to descriptions by other groups. [56,93]

The HLA-DQ5 allele is in linkage disequilibrium with several HLA-DR alleles including DR1, DR10, DR14, and DR16. Although DQ5 as a group was significantly increased in MuSK MG, no significant association was found for the DR1-DQ5, DR10-DQ5, and DR16-DQ5 haplotypes as shown in Table 4.2. The following DQ5 haplotypes can be observed: DQB1*0501 with DR1 and DR10, DQB1*0502 with DR16 and DQB1*0503 with DR14. These DQ5 molecules differ in their β-chains, which may influence antigen presentation. The association with DR14 is highly significant with an OR of 18.8, and we found one patient with DR14-DQ6, an extremely rare haplotype in white Dutch people, being absent in 321 healthy controls. Therefore, we speculate that the association is explained by the presence of the DR14-DQ5 haplotype rather than by DQ5 as a group.
DR14 is also associated with pemphigus vulgaris in non-Jewish white and Pakistani patients. [121,122] The DR14-DQ5 haplotype is even linked to the presence of pemphigus-specific anti-desmoglein 3 antibodies in relatives of the Pakistani patients. Interestingly, in both pemphigus foliaceus and vulgaris, autoantibodies directed to desmoglein 1 and 3 are mainly of the IgG4 subclass during periods of disease activity. [123] A predominance of the non-complement fixing IgG4 isotype is also found in MuSK MG. [93] In contrast, autoantibodies in AChR MG are mainly IgG1 and IgG3 isotypes and cause complement-induced damage to the postsynaptic membrane. The association of MuSK MG and the HLA DR14-DQ5 haplotype adds to the previously described differences in clinical appearance and IgG subclass distribution of antigen-specific antibodies between MuSK MG and AChR MG, suggesting a different immunopathogenesis for both diseases.

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V

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

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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).

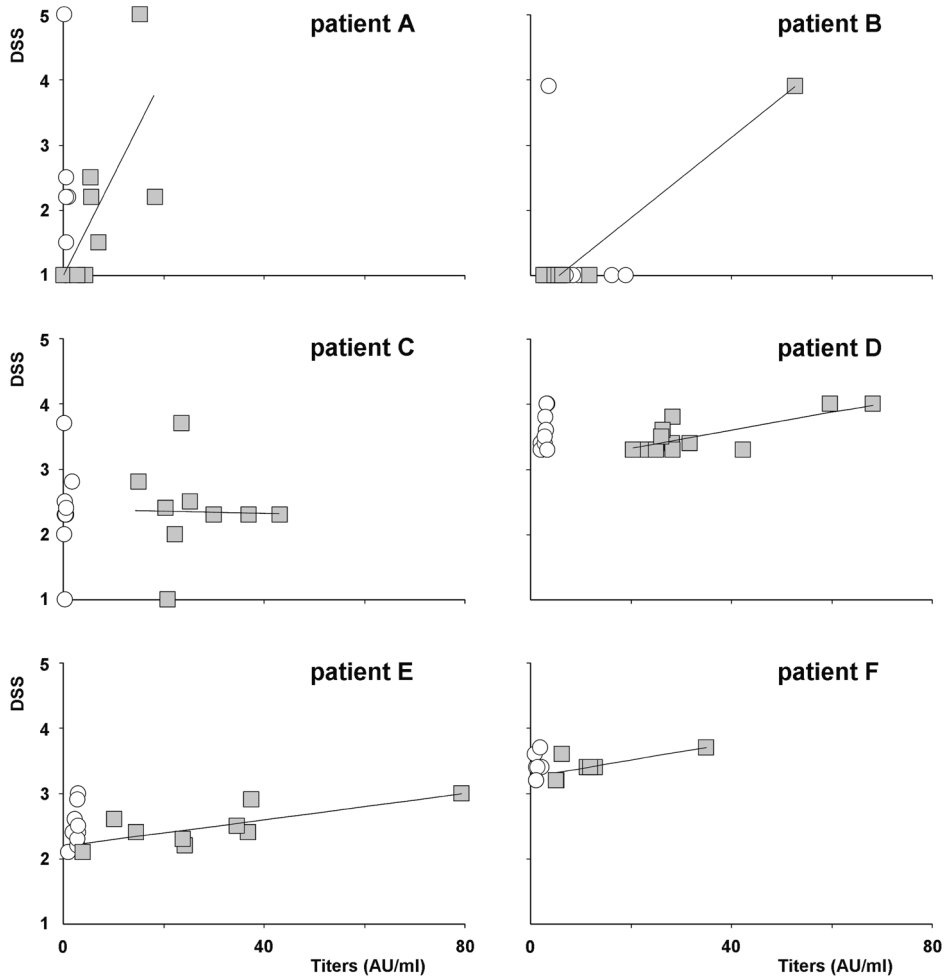

Figure 5.1 The Disease Severity Score (DSS) versus adjusted titres of anti-MuSK specific IgG1 and IgG4 in arbitrary units (AU) per ml in 6 patients
○ = IgG-1, □ = IgG-4

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.

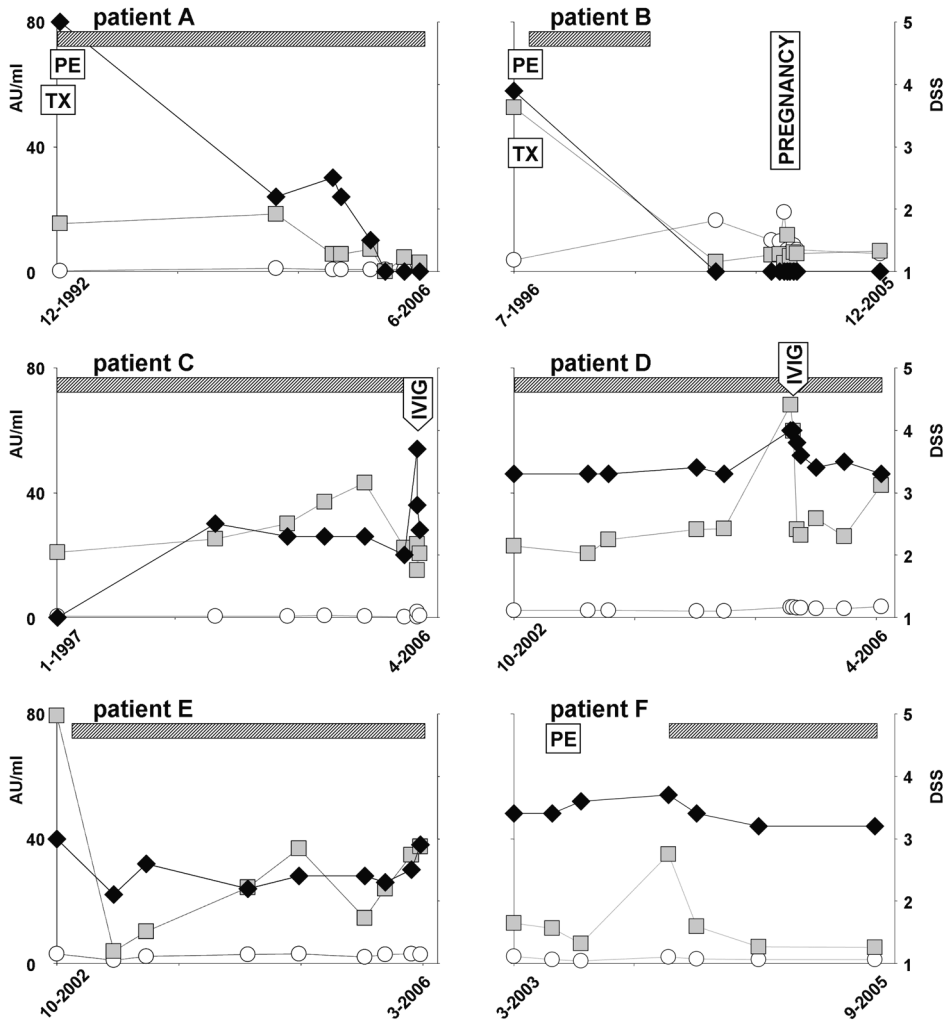

Figure 5.2 Course of Disease Severity Score (DSS) and adjusted anti-MuSK specific IgG1 and IgG4 titres in AU/ml during follow-up

○ = 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.

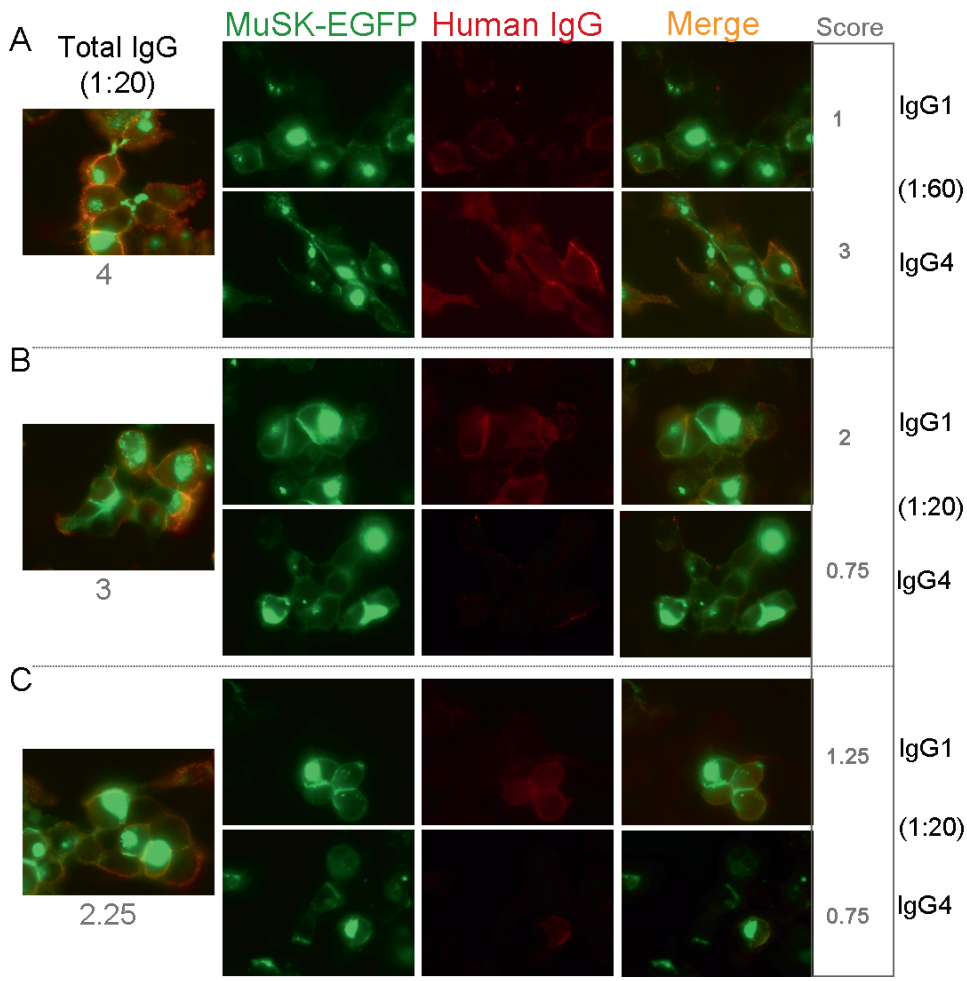

Figure 5.3 Specific binding of IgG antibodies (red) to MuSK-EGFP on HEK cell surface (green). A switch from IgG4 to IgG1 anti-MuSK antibodies is shown in three different serum samples from patient B, collected when the patient was severely affected in 1996 (A), and during clinical remission in 2001 (B), 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.

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VI

No auto-antibodies against synaptic ErbB in seronegative myasthenia gravis

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Abstract

In myasthenia gravis (MG), the neuromuscular transmission is impaired mainly by auto-antibodies against the acetylcholine receptor (AChR). In about 5% of the MG patients, however, the auto-antigen is still unknown. We investigated whether these seronegative MG patients (SNMG) have auto-antibodies against ErbB proteins, which influence the AChR density at the NMJ. Using Fluorescence-activated cell sorting (FACS) and dot blotting analysis of membrane extracts from transfected human embryonic kidney (HEK) cells, we were unable to detect anti-ErbB antibodies in sera of SNMG patients. The results do not support a role for ErbB receptors as auto-antigen in SNMG.

Introduction

Myasthenia gravis (MG) is an acquired autoimmune disease that affects the synaptic transmission between motor neurons and skeletal muscle cells. In about 85% of generalised MG patients, autoantibodies are present against the acetylcholine receptor (AChR), which is located at the postsynaptic membrane of the neuromuscular junction (NMJ). [9] In about 5% of the generalised MG patients the muscle-specific kinase (MuSK) is the target of the autoantibodies while antibodies to AChR are absent. [54] The remaining patients with generalised MG without detectable autoantibodies show symptoms that are similar to those presenting with anti-AChR positive MG. [134] These seronegative MG (SNMG) patients improve after immunosuppressive treatment, which strongly suggests that SNMG also has an autoimmune origin. [111,135] Approximately half of the SNMG subgroup appears to have antibodies to the AChR as well, although probably only with low affinity. [136] In a study by Leite *et al.* anti-AChR antibodies could only be detected by assays where the AChRs were clustered on the cell surface instead of being in a solubilised form, as is the case in routine assays for anti-AChR antibodies. [44] However, in about 5% of MG patients the auto-immune target remains unknown. We therefore tested the possibility that ErbB proteins can act as auto-antigens in SNMG.

ErbB proteins are receptors for neuregulins and are involved in the local activation of synaptic gene expression, such as AChR. [137,138] The ErbB receptor family consists of four members: ErbB1 (also known as the epidermal growth factor receptor or HER1), ErbB2 (also known as HER2 in humans and Neu in rodents), ErbB3 (or HER3) and ErbB4 (or HER4). In skeletal muscle, ErbB3 is expressed mainly at the terminal Schwann cells, whereas ErbB2 and ErbB4 are located postsynaptically in the depth of the folds. [137,139,140] Mutant mice lacking neuregulin or ErbB genes die early in embryogenesis due to defects in cardiac and neural development, but hemizygous mice are viable. [141,142] Mice hemizygous for the neuregulin-1 gene have a reduced concentration of AChRs and show myasthenic symptoms [143,144], while mice hemizygous for the ErbB2 or ErbB3 gene show modest but significant postsynaptic defects, but no clear cardiac defects. [145] As transmembrane proteins, ErbB receptors are potentially accessible to antibodies and could act as autoantigens in an antibody-mediated immune response. Therefore, we studied the presence of antibodies to ErbB receptors in patients with myasthenia gravis without antibodies to AChR or MuSK.

Materials and methods

Patient selection

Patients with generalised MG without anti-AChR antibodies were included from a nationwide study as described previously. [115] The diagnosis of generalised MG was based on acquired and clinically confirmed fluctuating weakness of muscles not restricted to the external eye muscles, the m. levator palpebrae or the m. orbicularis oculi. Absence of antibodies to the AChR in standard radioimmunoassays (RIA) was required more than once during periods of clinical weakness. The diagnosis was considered to be supported by a clinical response to intravenous acetylcholinesterase inhibitors or by electromyography if a decrement of the compound muscle action potential of >10% was found during repetitive nerve stimulation or if single-fiber electromyography had shown an increased jitter or blocking.

All patients were re-examined by a single investigator (EHN) for standardised history taking and physical examination. The clinical condition during the first two weeks and at maximum disease severity was classified according to the MGFA Clinical Classification. [113] Serum from all patients was retested for antibodies to the AChR, MuSK and voltage-gated calcium channels in standard RIA (RSR Ltd., UK). DNA of SNMG patients was tested for the rapsyn N88K mutation known to cause a late-onset congenital myasthenic syndrome. [45] Of the 62 patients with SNMG originally described in our epidemiological study, seven were lost to follow-up, while no informed consent was obtained from six, one patient had died and one 16-year-old girl had become AChR antibody positive three years after the onset of the symptoms. The remaining 47 patients were clinically re-examined. Subsequently, five patients were excluded because the symptoms were incompatible with generalised MG (n=2), or because of the presence of a N88K rapsyn mutation (n=1), or comorbidity in the form of severe Cushing's disease before the onset of MG symptoms (n=1), or finally because one patient appeared to have presented fluctuating weakness ever since birth (classified as congenital myasthenic syndrome, n=1). Clinical characteristics of 42 patients with generalised SNMG are summarised in Table 6.1. Muscle weakness was mild or moderate, and more of the limb-girdle type (MGFA class IIa or IIIa). However, a few patients suffered from oculobulbar and axial weakness, even requiring intubation in two cases (MGFA class V). The diagnosis was supported by repetitive nerve stimulation in 14 patients, by single-fiber electromyography in 17 patients and by IV acetylcholinesterase inhibitor in eight patients. In one patient, diagnostic procedures were incomplete. Only repetitive nerve stimulation was performed but negative. In the remaining two patients, results of repetitive nerve stimulation, single-fiber electromyography and IV acetylcholinesterase inhibitor were all negative. In all three patients however, clinical symptoms had responded to immunosuppressive therapy.

Table 6.1 Characteristics of 42 SNMG patients

Women - *n* (%)	28	(67)
Age at onset - median (range) in yrs	38.1	(5.9 - 66.8)
Age at onset below 16 yrs - *n* (%)	5	(12)
Follow-up - median (range) in yrs	10.4	(0.6 - 45.5)
Caucasian - *n* (%)	40	(95)
MGFA at onset		
- I - *n*	19	
- II (a *vs*. b) - *n*	23	(14 *vs*. 6)
MGFA at maximum or before thymectomy for French patients		
- II (a *vs*. b) - *n*	20	(11 *vs*. 2)
- III (a *vs*. b) - *n*	15	(9 *vs*. 4)
- IV (a *vs*. b) - *n*	3	(0 *vs*. 2)
- V - *n*	2	
Interval onset to maximum - median (range) in yrs	2.1	(0 - 40.5)
Diagnosis supported by		
- A. Electromyography - *n* (%)	31	(74)
- B. Response to IV AChE inhibitors - *n* (%)	8	(19)
- Neither A or B - *n* (%)	3	(7)
Therapy		
- Oral AChE inhibitors - *n* (%)	41	(98)
- Immunomodulative therapy - *n* (%)	26	(62)
- Oral immunosuppression - *n* (%)	23	(55)
- Thymectomy (with follicular hyperplasia) - *n*	11	(3)
Interval onset to start of immunomodulation - median (range) in yrs	2.4	(0 - 40.5)

Fluorescence-activated cell sorting (FACS)

Sera from 42 SNMG patients were screened for anti-ErbB-IgG using FACS. For this purpose murine 32D hematopoietic cells transfected with distinct combinations of human ErbB receptors were used. In the present study we used 32D13 cells (expressing both ErbB1 and ErbB3), 32D23 cells (expressing both ErbB2 and ErbB3) and 32D24 cells (expressing both ErbB2 and ErbB4). Transfected 32D cells were cultured in RPMI 1640/glutamax-I, containing penicillin (100 U/ml), streptomycin (100 µg/ml), 10% fetal calf serum (FCS), 50 µM β–mercaptoethanol, recombinant mouse IL-3 (0.5 ng/ml, R&D systems, Minneapolis, MN, USA), geneticin (G418, 600 µg/ml) and hygromycin B (for 32D13 and 32D23 cells, 400 µg/ml). The cells were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO2. High density 32D cells were harvested and resuspended in washing buffer (PBS containing 1% BSA/1% FCS/0.2% NaN3). Subsequently cells (0.15x106) were incubated with sera diluted to a final concentration of 1:10, 1:20 and 1:40 in washing buffer for 1 hour while gently shaking. Cells were then washed five times with washing buffer and incubated for 30 min with rabbit anti-human-IgG-FITC conjugate (1:20, Dako, Glostrup, Denmark). As a negative control, normal human serum (NHS; healthy volunteers) was

analysed according to the same protocol. The expression of ErbB2 was checked with a mouse monoclonal anti-c-ErbB2 (clone L26, Immunologic, Duiven, The Netherlands) and goat anti-mouse Ig-APC conjugate (BD Pharmingen, San Jose, CA, USA). Cell lines 32D23 and 32D24 were positive for ErbB2 staining in contrast to 32D13 which was negative. FACS analysis was performed using a BD Biosciences FacsCalibur flowcytometer and the mean fluorescence intensity (MFI) was calculated with CellQuestPro software (BD Biosciences, San Jose, CA, USA). The MFI of each sample was normalised by dividing the obtained fluorescence values by the mean of the corresponding NHS group. The mean value of NHS plus two and three times the standard deviation was used as cut off value.

Dot blotting

Sera from 42 SNMG patients were also screened for anti-ErbB-IgG using dot blotting. For this, we used human embryonic kidney (HEK) 293 cells, transiently transfected with expression vectors for human ErbB2 and ErbB4 genes using the Profection mammalian calcium phosphate transfection system (Promega Corporation, USA). These vectors were a kind gift of Dr. L. Schaeffer from the 'Laboratoire de biologie moleculaire et cellulaire' in Lyon, France. Transfected cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with glucose and sodium pyruvate (Gibco, Invitrogen Corporation, Breda, The Netherlands) supplemented with 10% FCS (Bodinco, The Netherlands), 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Gibco). The cells were cultured in a 37°C humidified atmosphere containing 95% air and 5% CO2. After 72 hours, membrane proteins were isolated from the cells with a 0.6% cholate extraction buffer, containing 0.01 M NaN3 (Acros Organics, Geel, Belgium), 0.01 M EDTA (Sigma-Aldrich, St-Louis, USA), 0.01 M iodoacetamide (Sigma), 0.1 mM phenylmethylsulphonyl fluoride (Sigma) and 0.6% cholate (Sigma) in PBS. The protein concentration was measured using the Bio-Rad system (Bio-Rad, California, USA). To verify the expression of ErbB2 and ErbB4 in transfected HEK cells, Western blotting was performed as described before using rabbit anti-ErbB2 antibody (C-18) (sc-284, Santa Cruz Biotechnology Inc., USA) or of rabbit anti-ErbB4 antibody (C-18) (sc-283, Santa Cruz).[146]

To detect anti-ErbB antibodies in sera of SNMG patients, 5 µl of ErbB transfected HEK 293 cell extract (0.4 mg/ml protein concentration) and non-transfected cell extract (0.4 mg/ml) were applied in three dots on a Protran nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Following blocking in Odyssey blocking buffer (OBB; LiCor Westburg, Leusden, The Netherlands) diluted in PBS (1:1) (OBB/PBS), membrane strips were separately incubated with rabbit anti-ErbB2-antibody or anti-ErbB4 antibody (positive controls), diluted 1:1000 or 1:1500 respectively in OBB/PBS containing 0.5% goat serum (GS) (i.e. OBB/PBS/GS), with OBB/PBS/GS (negative control), with patient serum diluted 1:1000 in OBB/PBS/GS, or with NHS diluted 1:1000 in OBB/PBS/GS. Then, strips were

washed three times with PBS 0.1% Tween (Merck), and incubated with IRDye 800 goat anti-rabbit IgG (Rockland Immunochemicals), diluted 1:3700 in OBB/PBS/GS, or with IRDye 800 goat anti-human IgG (Rockland Immunochemicals), diluted 1:10000. Following the washing steps, strips were scanned with the Odyssey scanner (LiCor Westburg) at 800 nm. Finally, ImageJ software (http://rsb.info.nih.gov/ij/) was used to quantify the integrated intensities of the dots. The mean integrated intensity of the background was subtracted. To correct for the non-specific binding of antibodies to other proteins than ErbB2 or ErbB4, the ratio was calculated between mean intensities of dots of transfected cell extract and dots of non-transfected cell extract. These ratios were normalised by dividing them by the mean of the NHS ratios. The mean of NHS plus two and three times standard deviation was used as cut off value.

Statistical analysis

SPSS Software 17.0 was used to perform statistical analyses. Comparison between non-normally distributed values was performed using the non-parametric Mann-Whitney test. A two-sided probability value <0.05 was considered statistically significant.

Results

Results of the anti-ErbB antibody measurement by FACS are summarised in Figure 6.1. The median MFI of SNMG patients was not significantly higher than that of healthy controls, for none of the three cell lines tested. In addition, no individual value of an SNMG patient or a healthy control exceeded the cut off value, calculated as the mean plus three times SD of the corresponding NHS group (data not shown). These results show that there are no detectable levels of antibodies against ErbB1, ErbB2, ErbB3 and ErbB4 in this cohort of SNMG patients.

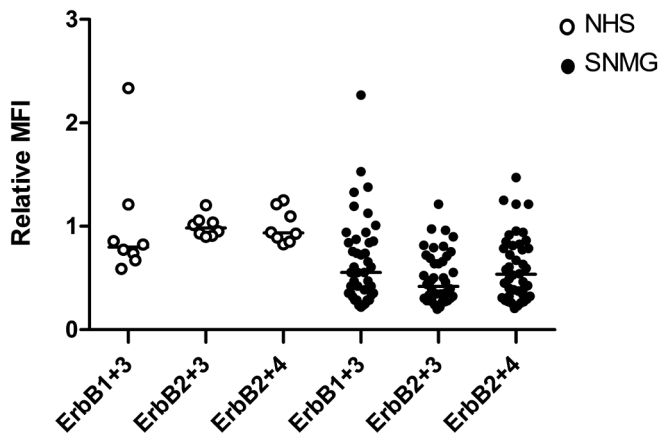

Figure 6.1 Detection of anti-ErbB2/3/4 antibodies by FACS
Sera from 42 SNMG patients (SNMG) were screened for anti-ErbB-IgG by flow cytometry. 32D13 cells (expressing ErbB1+3); 32D23 cells (expressing ErbB2+3); and 32D24 cells (expressing ErbB2+B4) were incubated with serum from patients (solid circles) or normal human serum (NHS) (empty circles). Values are normalised with the mean MFI of the corresponding NHS group. Horizontal full lines represent the median MFI per group. The Mann-Whitney test did not show a significant increase in median MFI in SNMG patients compared to NHS.

Results of the dot blotting analysis are shown in Figure 6.2. The high transfection efficiency of HEK cells with ErbB2 and ErbB4 cDNA was verified by Western blotting (Figure 6.2A). Low amounts of ErbB2 receptors were also endogenously present in non-transfected HEK cells, whereas ErbB4 receptors were undetectable in non-transfected HEK cells. Figure 6.2B shows representative dot blots, where non-transfected and transfected HEK cell extracts were incubated with SNMG patient serum (Figure 6.2B, row 1), normal human serum (Figure 6.2B, row 2), monoclonal anti-ErbB2 antibody (Figure 6.2B, row 3) and monoclonal anti-ErbB4 antibody (Figure 6.2B, row 4). To correct for unspecific antibody binding to endogenous HEK proteins, the mean integrated intensity of transfected cells were divided by the mean integrated intensity of non-transfected cells for each sample. The results are summarised in Figure 6.2C. The median fluorescence ratio in SNMG patients was significantly lower than that of both positive controls (monoclonal anti-ErbB2 antibody ($p<0.001$) and monoclonal anti-ErbB4 antibody ($p<0.001$)), but did not differ from the median ratio of healthy individuals. In addition to comparing median values of the ratio of the different groups, no individual ratio of an SNMG patient or a healthy person exceeded the cut off value.

To study the possibility that the negative results were due to low sensitivity of the techniques, we validated the sensitivity of the dot blotting protocol (Figure 6.2D). Anti-AChR antibodies were measured in AChR-MG patients, of whom the anti-AChR antibody titre had been determined by RIA before. Anti-AChR antibodies were detectable using the present dot blotting technique, although the dot blotting was less sensitive than the RIA (data not shown).

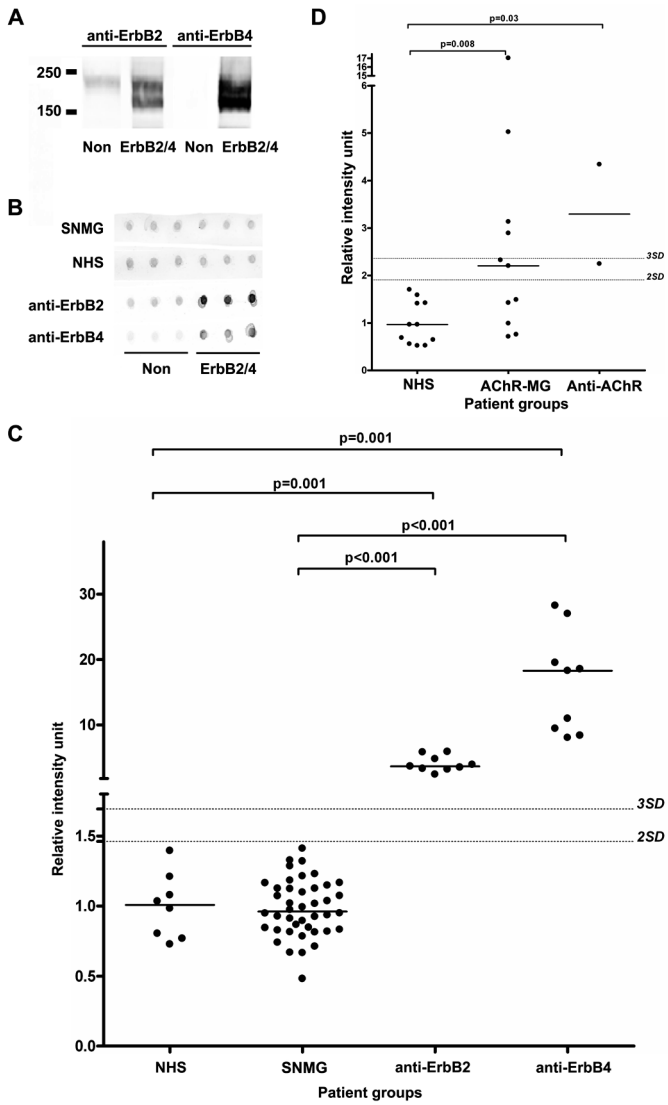

Figure 6.2 Detection of anti-ErbB2/4 antibodies by dot blotting analysis

A) Expression of ErbB2 and ErbB4 in non-transfected (non) and transfected (ErbB2/ErbB4) HEK 293 cells, measured by Western blotting. B) Representative dot blots spotted with non-transfected and transfected HEK 293 cell extracts were incubated with SNMG patient serum (SNMG), normal human serum (NHS), anti-ErbB2 positive control antibody or anti-ErbB4 positive control antibody. C) Dot blot results of anti-ErbB antibody measurement in SNMG patients. Each dot corresponds to the ratio between the mean intensity of triplicate measurements of transfected HEK cells and the mean intensity of the non-transfected HEK cells. Horizontal full lines represent the median value of the ratio per group, dashed lines show cut off value calculated as the mean of NHS plus two and three times the standard deviation. D) Dot blot results of anti-AChR antibody measurement in AChR-MG patients. Horizontal full lines represent the median ratio per group, dashed lines show cut off value calculated as the mean of NHS plus two and three times standard deviation. The *p*-values were obtained by the Mann-Whitney test and shown when <0.05.

Discussion

Various studies have identified ErbB receptors, which are present at the NMJ, as potential candidates for the autoimmune target in SNMG patients, particularly since myasthenic symptoms have been described in ErbB mutant mice. [141-145] The antibody tests for ErbB in the sera of the 42 SNMG patients were all negative. This strongly suggests that ErbB does not have a role as autoantigen in MG.

The present negative finding could be explained if the sensitivity of our assays for anti-ErbB would have been too low. However, we successfully used an analogue FACS analysis to demonstrate the presence of anti-MuSK antibodies, which confirms the sensitivity of the technique (data not shown). The dot blot assay was not able to detect anti-AChR antibodies in all the AChR-MG patients, and thus might be too insensitive for detection of low anti-ErbB antibody titres. A second possibility is that the autoantibodies would only be able to bind to ErbB proteins when clustered together in the NMJ, as shown earlier for a subset of anti-AChR antibodies. [44] A third, more remote, possibility is that patients with a low titre of putative antibodies might bind to the ErbB antigens with very high affinity, thereby leaving the blood serum depleted of free antibody.

If, on the contrary, anti-ErbB antibody would have been present in patients it is questionable that they would actually have caused MG-like symptoms. ErbB2 gene amplification and protein overexpression are known to be critical for the development of malignancies such as breast cancer. For more than ten years, such patients are frequently treated with trastuzumab (Herceptin), a humanised IgG1 monoclonal antibody directed against ErbB2. [147-149] Distinct side effects can occur during treatment with trastuzumab but, as far as we know, myasthenic muscle weakness in patients undergoing this therapy has not been reported, arguing against a possible myasthenic effect of anti-ErbB2 antibodies. No reports specifically analysed these patients for myasthenic syndromes, leaving the possibility that milder forms of the disease might have been missed in these patients.

In conclusion, we could not detect anti-ErbB antibodies in sera of SNMG patients, and therefore further research is needed to identify unknown autoantigens. Interestingly, the thymic transcriptome of SNMG patients is characterised by a down-regulation of several genes involved in synaptic transmission, including neurotransmitter receptors. [150,151] These genes could potentially correspond to autoimmune targets, and would be worthwhile to focus on while seeking other autoantigens responsible for SNMG.

Acknowledgements

The authors thank Ingrid Hegeman for technical support and Catelijne Stortelers for carrying out Western blot analyses for detecting the presence of anti-ErbB antibodies. This work was supported by a grant from the European Myastaid Project (LSHM-CT-2006-037833).

VII

A transient neonatal myasthenic syndrome with anti-MuSK antibodies

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Introduction

Some patients with myasthenia gravis (MG) have autoantibodies to muscle-specific kinase (MuSK) instead of the acetylcholine receptor (AChR). Anti-AChR antibodies may be transferred across the placenta causing a self-limiting neonatal myasthenic syndrome. We describe an infant with a similar disorder whose mother had MuSK MG.

Case report

In September 1994, the mother noticed unilateral ptosis and a feeling of generalised fatigue at age 13. Over the next months, she developed severe oculobulbar weakness, dyspnoea and weakness of the neck. Symptoms were fluctuating and unresponsive to acetylcholinesterase inhibitors. No antibodies to the AChR were found. In 1995, a normal thymus was removed. High doses of prednisone had little effect. In 1997, plasmapheresis induced a partial remission and this became her regular therapy for several years. In 2004 anti-MuSK antibodies were found. She had two first trimester miscarriages while using pyridostigmine and prednisone in September and December 2004. In May 2005, she became pregnant using only prednisone 20 mg on alternating days. This pregnancy was uneventful without large fluctuations of her myasthenic symptoms.

In February 2006, at 38+1 week of gestation, she unaidedly delivered a boy in head position. Apgar score was 10 after 1 and 5 minutes. Birth weight was 3190 gram (P25). Physical examination was unremarkable. After 8 hours, however, drinking became difficult. After 16 hours, he showed mild generalised hypotonia with diminished facial expression and a weak cry. Thirty-two hours after birth, examination showed paucity of general movements, a tent-shaped mouth and chest retractions. After 10 minutes of examination, weakness increased, leading to an immobile frog position (Figure 7.1). Tube feeding was initiated. Intramuscular neostigmine gave a temporary improvement of symptoms and oral pyridostimine 0.2 mg every 3 hours was started. On day 4, the amount of tube feeding could gradually be decreased. Quantities of spontaneously ingested bottle feedings increased from 5 mL on the 4th to 35 mL on the 8th and to 75 mL on the 13th day. On day 11, examination showed only a mild head lag. On day 15, he was discharged, spontaneously drinking his bottles of 7 times 80 mL. On day 22, neurological examination was unremarkable. Further growth and development were normal.

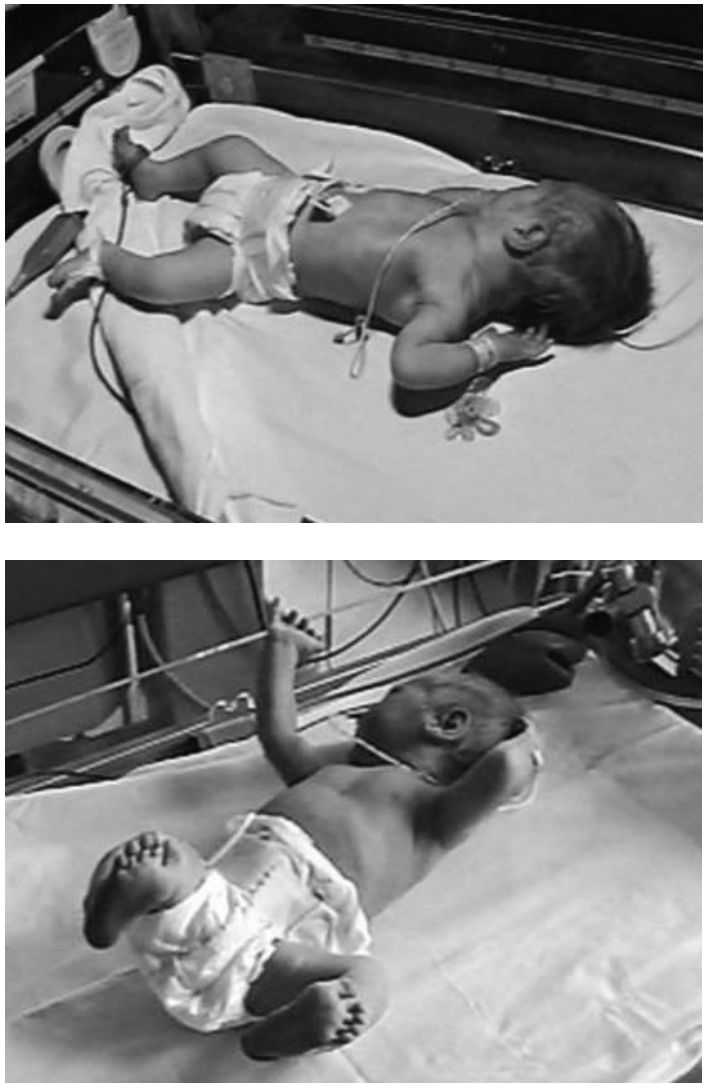

Figure 7.1 Neonatal myasthenic weakness on day 3 (top) and remission on day 6 (bottom)
On day 3 the infant shows severe muscle weakness lacking the power to overcome gravity. On day 6, normalisation of muscle strength was observed. Parental consent was obtained for publication.

Maternal and neonatal anti-MuSK specific IgG1 and IgG4 levels were measured by ELISA, [93] using monoclonal antibodies anti-Human IgG subclasses (IgG1, MH 161-1, Sanquin, Amsterdam, The Netherlands, and IgG4, NI315, Nordic, Tilburg, The Netherlands) and alkaline phosphatase (AP) labelled Rabbit anti-Mouse Ig (1:750, Dakopatts, Glostrup, Denmark). Total IgG anti-MuSK titres were measured using AP labelled Goat anti-Human IgG (Biosource, Camarillo, CA, USA). The ratio of MuSK-specific total IgG versus subclass

IgG in pooled sera from MuSK MG patients containing predominantly one anti-MuSK specific IgG subclass yielded a factor enabling direct comparison of IgG1 and IgG4 anti-MuSK titres in these patients (Figure 7.2).

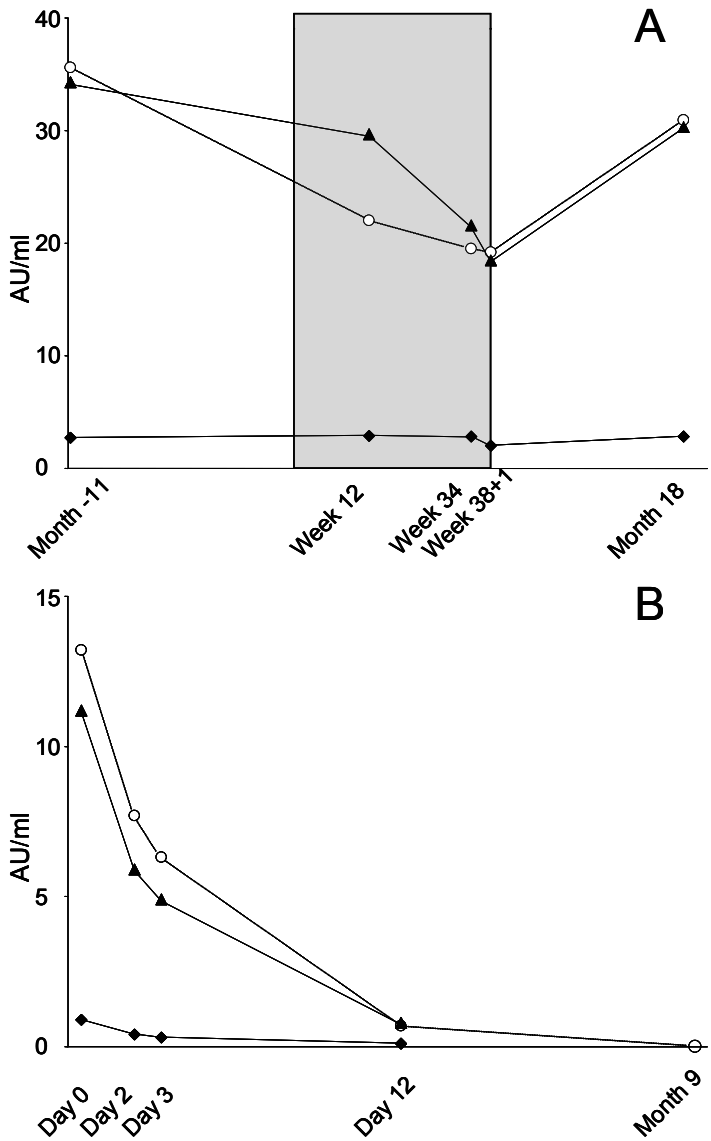

Figure 7.2 Course of maternal (A) and neonatal (B) titres of anti-MuSK specific IgG antibodies
Titres are expressed as arbitrary units per mL compared to an internal standard. The time scale is related to the day of conception (A) and the day of birth (B).
◯ = *total IgG,* ◆ = *IgG1,* ▲ = *IgG4,* □ = *Pregnancy*

Discussion

This case report shows that transient neonatal myasthenia gravis (NMG) is possible through cross placental transport of maternal anti-MuSK antibodies. The pathogenicity of the antibodies, that are mainly IgG4 in the patients presented here, is supported by the paralleled course of neonatal titres and clinical symptoms. NMG without anti-AChR antibodies has been described before the role of MuSK in seronegative MG became known. [50] In retrospect, anti-MuSK antibodies may have been involved in some of these patients. In AChR MG, 10 to 15% of the infants develop NMG although autoantibodies are transferred to nearly all infants. [152] Giving birth to an infant with NMG seems to enhance the risk of NMG in following newborns. [153] There are contrasting data whether the maternal anti-AChR antibody titre is predictive for the occurrence of NMG. Both healthy infants from mothers with high titres and affected ones from mothers with low titres have been described. [154,155] Active transport of IgG across the placenta favours IgG1 over IgG4, IgG3 and IgG2 and takes place from early in the second trimester. [156] The risk of NMG in IgG4 mediated MuSK MG could therefore be lower than in IgG1 mediated AChR MG. The delay between delivery and onset of symptoms is remarkable. Most probably, neuromuscular synapses have been exposed to maternal anti-MuSK antibodies in utero but the infant had an excellent start and fetal development was normal. In animals, extensive remodelling of synapses occurs in the weeks after birth and MuSK might play an essential role in this process. In view of the short symptom free period, one could also postulate the existence of a short lasting mechanism optimizing the first essential muscle contractions of the neonate, like an abundance of presynaptic acetylcholine or decreased acetylcholinesterase activity, counteracting the myasthenic effect of the antibodies. It should be noted that two earlier pregnancies ended in spontaneous abortions with unknown cause. Although these occurred in the first trimester, a pathogenic effect of anti-MuSK antibodies in utero cannot be excluded. In pregnant mothers with MuSK MG, fetal and neonatal development of the infants should be carefully observed.

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VIII

Pre- and postsynaptic neuromuscular junction abnormalities in MuSK myasthenia

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Abstract

Autoantibodies to muscle-specific kinase (MuSK) can cause myasthenia gravis (MG). The pathophysiological mechanism remains unknown. We report in vitro electrophysiological and histological studies of the neuromuscular junction in a MuSK MG patient. Low levels of presynaptic acetylcholine release and small miniature endplate potentials were found. This combination of pre- and postsynaptic abnormalities was supported by histology, revealing partially denervated postsynaptic areas, and some degeneration of postsynaptic folds. Results suggest that anti-MuSK antibodies reduce the stability of muscle-nerve contact.

Introduction

Autoimmunity against the neuromuscular junction leads to muscle weakness in myasthenia gravis (MG). Although the majority of patients are seropositive for autoantibodies against the postsynaptic acetylcholine receptor (AChR), a minority (<6%) have autoantibodies against muscle-specific kinase (MuSK). [54,157] MuSK is a transmembrane tyrosine kinase localised at the postsynaptic specialisation of the muscle cell membrane. [65,158] In conjunction with the proteins LRP4 [73,74] and Tid1, [79] it is involved in the development and maintenance of the neuromuscular junction, in particular the clustering of AChRs in the postsynaptic membrane through tyrosine phosphorylation, and in the localisation of acetylcholinesterase (AChE) in the basal lamina of the synaptic cleft through interaction with its anchoring protein ColQ. [65, 80] Although the molecular mechanism by which MuSK/LRP4/Tid1 regulate neuromuscular junction formation and maintenance has become increasingly clear, [159,160] the pathogenic role of anti-MuSK antibodies and the pathophysiological mechanisms by which they may cause muscle weakness in MuSK MG are less well understood. The high disease specificity of anti-MuSK antibodies, the correlation of their titre with muscle weakness severity, [161] and the experimental demonstration in myotubes that these antibodies are capable of reducing MuSK-dependent AChR clustering, [54,158] all suggest a pathogenic role. However, no clear histological abnormalities consistent with a disturbance of neuromuscular synaptic function have been found in biopsies of affected muscles. In particular, a reduction of AChR density was not found in morphological studies, in contrast to positive control tissue from AChR MG patients. [125,126] Despite these findings, a reduction in the amplitude of miniature endplate potentials (MEPPs) was observed in the only MuSK MG biopsy to date in which neuromuscular synaptic function was studied with microelectrode methods. [126] MEPPs are the unitary postsynaptic responses resulting from spontaneous presynaptic release of single acetylcholine (ACh) quanta and their amplitude is largely determined by the density of functional AChRs. MEPP amplitude reduction is an established electrophysiological hallmark of neuromuscular junctions of AChR MG patients. [162] In view of the apparent inconsistency between electrophysiological and morphological observations at biopsied MuSK MG neuromuscular junctions and the very limited data reported so far, it is important to study function and morphology in additional MuSK MG patients. In this study we describe in vitro electrophysiological and histological aberrations of the neuromuscular junction in a patient with acquired MuSK MG.

Methods

Muscle biopsy

Parasternal intercostal muscle tissue was obtained under local anaesthesia using lidocain as part of a study of seronegative MG performed at the University Medical Center of Utrecht in 1994 and 1995. Approval from the local medical ethics committee and informed consent from the patient were obtained. The histological examination took place at the same hospital. Immediately after obtaining the biopsied material, part of the biopsy (a few centimetres long) was transported in 250 ml of pre-oxygenated Ringer's medium to the Leiden University Medical Center for in vitro electrophysiology studies.

Endplate electrophysiology

The in vitro electrophysiology studies were performed as described previously. [162] In brief, muscle fibers in Ringer's medium containing 2 mM Ca^{2+} and 1 mM Mg^{2+} at 26-28°C were impaled with a glass microelectrode, connected to standard electrophysiological and digitising equipment. In the MuSK MG biopsy, synaptic signals were recorded from a sample of 28 endplates. At each endplate, 4-26 MEPPs were recorded, from which the mean endplate value was calculated. At each of the 28 studied endplates, 28-42 endplate potentials (EPPs) were recorded during 0.3-Hz electrical stimulations of an intramuscular nerve branch through a suction stimulation electrode, after allowing for some depolarisation to prevent muscle fiber action potentials (at resting membrane potentials of around -65 and more positive, EPPs at most human muscle fibers no longer trigger an action potential [162], presumably due to Na^+ channel inactivation). The mean resting membrane potential during EPP recordings was -67.2 mV. At each endplate a mean 0.3-Hz EPP amplitude was calculated from the amplitudes of the recorded EPPs. Whenever possible, EPPs were also recorded at 3- and 30-Hz nerve stimulation. Mean MEPP and EPP amplitudes at each endplate were normalised to a standard resting membrane potential of -75 mV and EPPs were corrected for nonlinear summation. The quantal content, which is the number of ACh quanta released per nerve stimulus, was calculated directly by dividing the normalised and corrected mean 0.3-Hz EPP amplitude by the normalised mean MEPP amplitude at each endplate. From the mean endplate values of each electrophysiological parameter at the 28 sampled endplates, an overall biopsy mean was calculated. Control data were obtained from biopsies of 4 non-neurological-diseased subjects (referred to as "controls") and 6 patients with AChR MG, [162] supplemented by the unpublished results of 17 more AChR MG patients and 3 more controls.

Endplate histology

Part of the biopsy specimen was pinned out to prevent shrinkage and fixed in a periodate-lysine-paraformaldehyde solution. For electron microscopy, part of this material was refixed in 2% glutaraldehyde solution, postfixed in 1% osmium tetroxide solution, dehydrated and embedded in Epon 812. Endplate-containing regions were located in semithin sections stained with toluidine blue. Ultrathin sections from selected regions were contrasted with uranyl acetate followed by lead citrate and viewed using an electron microscope (1200 EX. JEOL Ltd., Tokyo, Japan). Another part of the specimen was freshly frozen with isopentane cooled in liquid nitrogen. Longitudinal 50-µm-thick cryostat sections were stained with a silver-choline esterase method for intramuscular nerve fibers and endplates. [163]

Results

The patient

In 1994, a 29-year-old woman experienced fluctuating ptosis and diplopia. Symptoms improved upon administering neostigmine. Oral pyridostigmine was unsuccessful, causing only gastrointestinal side effects and muscle trembling. Anti-AChR antibodies were negative. Two months later, she experienced progressive dysphagia, dysarthria and weight loss. She became dyspnoeic after exercise and had to support her head when doing the housekeeping and her jaw when chewing. Neurological examination also revealed mild proximal weakness of her arms and legs. Anti-AChR antibodies were absent again. Tube feeding was initiated. Spirometry showed a reduced vital capacity (71% of predicted) and a very low maximal inspiratory pressure (MIP, 21% of predicted). No compound muscle action potential decrement was found upon repetitive stimulation of the ulnar, facial and accessory nerve. Stimulated single-fiber electromyography of the orbicularis oculi muscles revealed increased jitter and blocking. Neostigmine led to slight improvement of the MIP (35% of predicted), but also to generalised fasciculations and muscle cramps. Nine months after onset, an intercostal muscle biopsy was performed. She had then been off all medication for 3 months. After the biopsy, prednisone treatment resulted in marked clinical improvement. In 2002, a relapse occurred with neck extensor weakness, diplopia, and mildly impaired swallowing and chewing. At this point, anti-MuSK antibodies were found that could also be demonstrated in stored serum from 1994. No antibodies to the AChR or voltage-gated calcium channels were found. To exclude a genetic cause, the *MuSK* gene was sequenced, revealing three known single-nucleotide polymorphisms (rs1784573, rs2766999 and rs578430), but no mutations in the coding sequence.

In vitro electrophysiology

The mean MEPP amplitude of the MuSK MG biopsy was 0.33 ± 0.02 mV; that is, approximately 50% smaller than the group mean value of the controls (and below the lower limit of the range of their individual biopsy mean values), but within the range of biopsy mean values from AChR MG patients (Table 8.1).

Table 8.1 In vitro electrophysiology in MuSK MG and controls

	MEPP			EPP			Quantal content
	Amplitude (mV)	Risetime (0-100%, ms)	Frequency (min^{-1})	Amplitude (mV)	Risetime (0-100%, ms)	Decay Tau (ms)	Quantal content
Patient biopsy mean ± SE	**0.33 ± 0.02**	**2.0 ± 0.02**	**2.60 ± 0.33**	**5.78 ± 0.59**	**2.3 ± 0.11**	**5.5 ± 0.26**	**18.8 ± 1.7**
AChR MG group mean ± SE	**0.21 ± 0.02** (0.11-0.43)	**2.1 ± 0.13** (1.2-3.8)	**3.84 ± 0.35** (1.02-9.01)	**9.65 ± 0.60** (5.32-14.94)	**2.9 ± 0.14** (1.6-4.6)	**7.5 ± 0.55** (4.7-16.7)	**58.3 ± 3.7** (35.8-84.5)
Controls group mean ± SE	**0.63 ± 0.08** (0.38-1.05)	**2.7 ± 0.20** (1.9-3.5)	**4.05 ± 0.54** (1.48-6.14)	**12.89 ± 1.50** (8.87-16.84)	**2.9 ± 0.16** (2.5-3.6)	**5.2 ± 0.58** (3.5-7.6)	**28.4 ± 1.7** (24.0-35.3)
Selcen mean ± SE	**0.35 ± 0.02**						**31 ± 5.8**
Controls by Selcen	**1.00 ± 0.025**						**31 ± 1**

In vitro electrophysiology data for the MuSK MG patient (biopsy mean was calculated from mean values of parameters at n = 28 endplates), AChR MG patients (group mean from biopsy means of n = 23 biopsies, range of biopsy means shown in parentheses), and healthy controls (group mean from biopsy means of n = 7 biopsies, range of biopsy means shown in parentheses). The last two rows include data published from a MuSK MG patient recorded by Selcen *et al*. [126] Data expressed as mean ± SE (range of biopsy means).

The amplitude of EPPs evoked by 0.3-Hz nerve stimulation was 5.8 ± 0.6 mV; that is, a 55% reduction compared with the group mean value of controls (Figure 8.1A). The mean MEPP frequency was rather low (2.6 ± 0.33 min^{-1}), but still fell within the ranges of AChR MG patients and controls. The EPP rise time was slightly smaller than the lower limit of the control range, but fell within the AChR MG range. The EPP decay time constant was in the range of AChR MG and controls. Mean quantal content was 18.8 ± 1.7; that is, 34% lower than the group mean of the controls, 68% lower than the group mean of AChR MG biopsies, and clearly below the lower limits of the ranges of the individual biopsy mean values of both groups. Upon repetitive 3-Hz nerve stimulation at normal resting membrane potentials (about -80 mV), some endplates showed intermittent subthreshold EPPs and muscle fiber action potentials triggered by suprathreshold EPPs, as well as delayed action potentials triggered by just-above-threshold EPPs (Figure 8.1B). High-rate stimulation (30 Hz) at normal resting membrane potential resulted in an increasing occurrence of subthreshold EPPs during the stimulation (Figure 8.1C). The 30-Hz EPP recordings at somewhat depolarised fibers (*i.e.*, at membrane potentials where EPPs no longer triggered

muscle fiber action potentials) showed that the amplitude of the EPP declined during the stimulus train to 54 ± 3.6% (n = 28 endplates) of its initial value (Figure 8.1D), which is close to the control group mean value (60 ± 4.5%; n = 4 biopsies), but less pronounced than the EPP decline level of AChR MG patients (group mean 42 ± 1.4%; n = 23 biopsies), although being just within its range of individual biopsy mean values (29-55%).

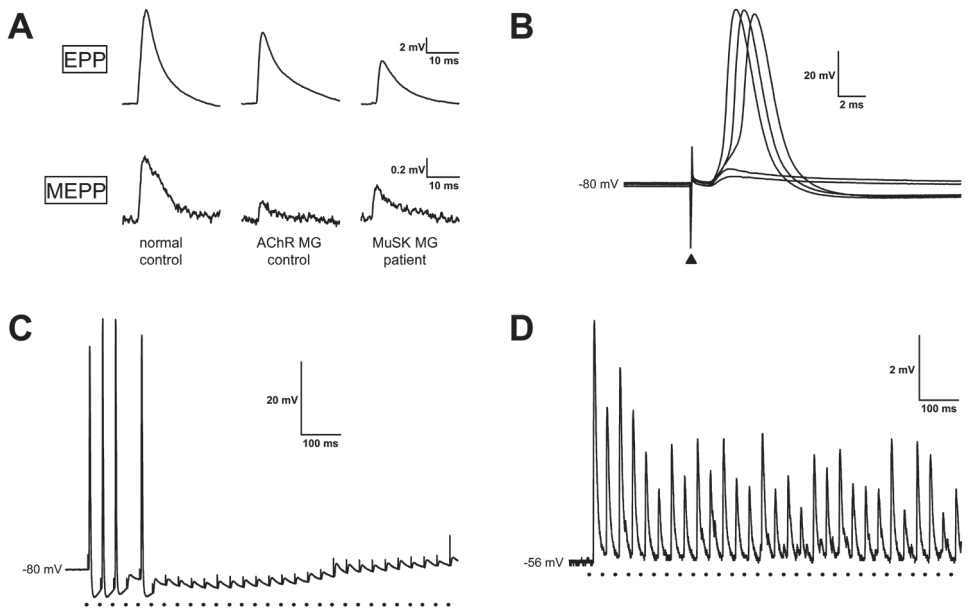

Figure 8.1 In vitro electrophysiology in MuSK MG
Electrophysiological signals recorded in vitro at neuromuscular synapses of the MuSK MG patient. For clarity, stimulus artifacts have been removed. (A) Representative examples of low-rate (0.3 Hz) evoked EPPs and spontaneous MEPPs. (B) Five superimposed example traces of responses recorded in the MuSK MG biopsy during 3-Hz stimulation. Two bare, apparently subthreshold, EPPs are shown and three muscle fiber action potentials, apparently triggered by suprathreshold EPPs, with variable delay relative to the moment of stimulation (black triangle). (C) Only the first few evoked EPPs trigger a muscle fiber action potential during high-rate (30 Hz) nerve stimulation (indicated by black dots). (D) Example trace of the rundown of EPPs at an endplate during 30-Hz stimulation.

Histology

Four endplates were available for analysis. One showed a postsynaptic area that was partially denuded of nerve terminal area and degenerated secondary postsynaptic folds (Figure 8.2A). Two other endplates had preserved postsynaptic areas, but were not covered by a nerve terminal (Figure 8.2B), although nearby one of these areas a preterminal nerve ending could be seen, surrounded by a Schwann cell, imposing as a nerve sprout. Z-disk streaming indicated myofibrillar degeneration (Figure 8.2C). One endplate appeared normal. Light microscopy revealed evidence of increased branching of intramuscular nerves (Figure 8.2D).

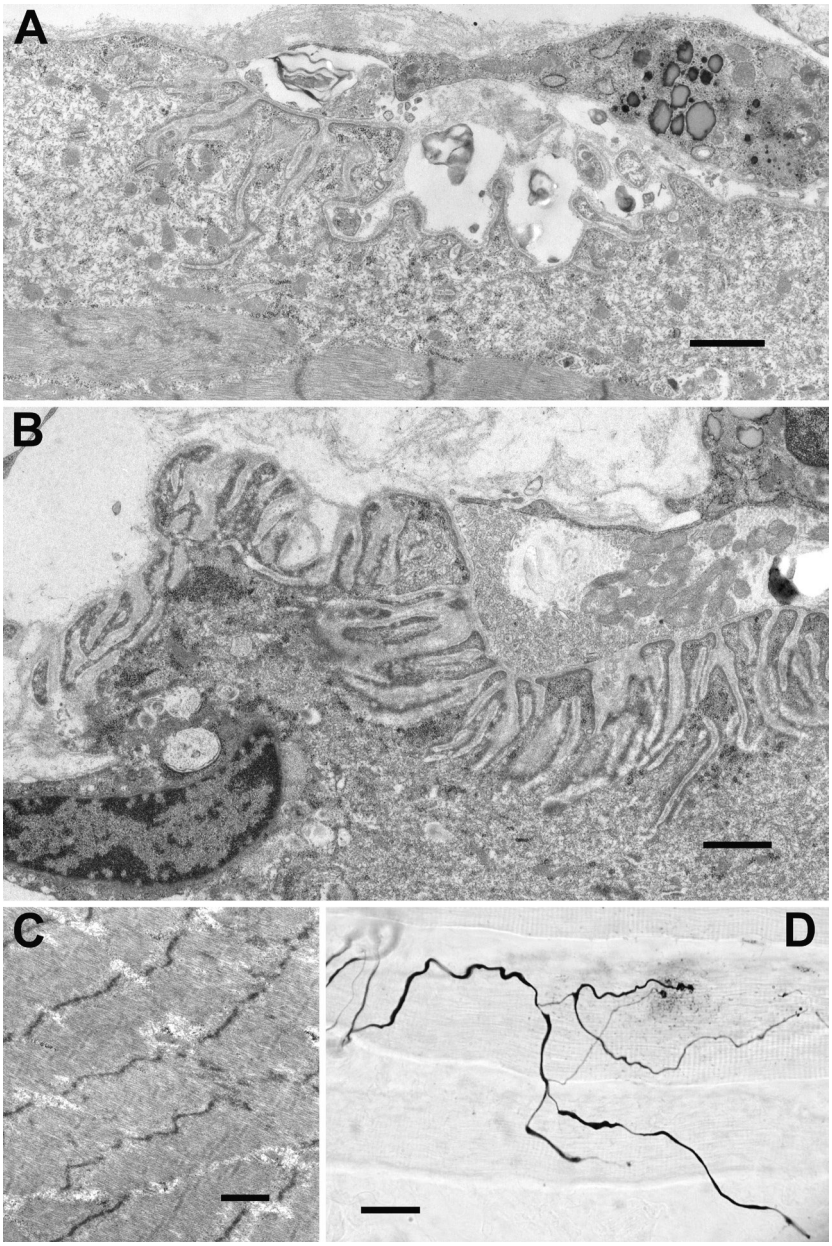

Figure 8.2 Histology in MuSK MG

Histological studies of the intercostal muscle biopsy. (A) Electron micrograph of a small profile of a nerve terminal (centre left) next to an area with degenerating postsynaptic folds. A Schwann-cell (upper right) contains abundant lipofuscin. (B) A nerve terminal (right) adjacent to a denuded area with conserved secondary clefts. Terminals contain translucent areas, sometimes with lipid remnants. (C) Z-disk streaming compatible with myofibrillar degeneration, which is also seen in the lower left corner of A. Bars in A, B and C = 1µm. (D) Light micrograph of silver-choline esterase staining for nerve fibers and endplates showing increased branching of intramuscular nerve fibers. Bar = 20µm.

Discussion

The course of symptoms, with spontaneous fluctuations and a response to immunosuppressive therapy, is consistent with that of an acquired autoimmune disorder of neuromuscular transmission. This could be confirmed by single-fiber electromyography in vivo, revealing an increased jitter and blocking, and by the presence of anti-MuSK antibodies in serum. Nine months after onset of symptoms, the biopsy was taken from a clinically affected muscle, as judged from the respiratory symptoms and the low spirometry values. At that time, no immunosuppressive therapy had been given. The patient had also not been treated with acetylcholinesterase inhibitors in the prior months.

In vitro electrophysiology showed small MEPPs of about half the control size. This is compatible with a postsynaptic defect, and has been unambiguously demonstrated at biopsied neuromuscular junctions of AChR MG patients. [162,164,165] Reduced MEPPs were found in another electrophysiological study of intercostal muscle endplates of a MuSK MG patient, [126] and in a preliminary animal study using active immunisation of mice with the extracellular domain of human MuSK. [157] Finally, passive immunisation of mice using serum obtained from anti-AChR-antibody-negative MG patients who later proved to be positive for anti-MuSK antibody, also led to a reduction of MEPP amplitude. [51,157] Thus, MEPP amplitude reduction seems to emerge as a general MuSK MG feature.

In another active immunisation study rabbits were immunised with an extracellular MuSK portion. Muscle weakness clearly developed and AChR density, as determined with fluorescence labelling, was greatly reduced. [166] However, such a reduction was not found in human morphological studies, [125,126] or in the passive transfer study of mice, as determined by radiolabelling. [51] This is a puzzling finding and suggests an effect of antibody binding to MuSK on AChR function.

Importantly, whereas in AChR MG an increase in quantal content can be observed as a compensatory presynaptic mechanism resulting from retrograde signalling from muscle fiber to nerve terminal, [162,165] the biopsy mean quantal content at the endplates of our study patient was 68% below that of the group mean of AChR MG patients, well below the lower limit of the range of biopsy means within this group, and even lower than in controls. In the MuSK MG patient described by Selcen *et al*., no reduced quantal content was found. [126] The quantal content in that study was calculated with the "variance method" (*i.e.*, from fluctuations of EPP amplitudes in preparations that were treated by d-tubocurarine to lower EPPs to subthreshold amplitude), instead of with the "direct method" without pharmacological manipulation, as applied here. The variance method is known to yield overestimated quantal content values, [162,167] and thus the value calculated by Selcen *et al*. may actually have been somewhat lower. Another important difference between the Selcen *et al*. study and our study is that the biopsy of their patient was taken later in the course of the disease, during a period

of relatively stable clinical symptoms, and in the presence of muscular atrophy, unlike the present case presented. In their accompanying morphological analyses, the presynaptic and postsynaptic neuromuscular junction ultrastructure seemed preserved.

A presynaptic defect might in theory result from a direct action of anti-MuSK antibodies on the presynaptic nerve terminal. However, neuronal presence of MuSK is unlikely,[61] although it cannot be completely excluded.[168] It is more likely that the autoimmune attack on MuSK in the postsynaptic membrane interferes, either directly or indirectly, with synaptic homeostatic and stabilisation pathways, with ensuing disturbance of retrograde signalling causing the presynaptic alterations. The observed pre- and postsynaptic defects at endplates in the MuSK MG biopsy led to failure of the neuromuscular transmission as indicated by small EPPs that became subthreshold, especially at high-rate stimulation of the motor nerve. The histological data accompanying our in vitro electrophysiological analyses must be regarded as qualitative and do not allow firm conclusions, in view of the large variability between endplates and the low number of observations we were able to make, due to the limited material. However, the observation of endplates (partially) lacking nerve terminals in combination with degeneration of postsynaptic folds and myofibrils supports the combination of pre-and postsynaptic abnormalities found in the microelectrode studies. Reduced MEPP amplitudes and quantal content have also been described in amyotrophic lateral sclerosis.[169] In patients with this disorder, a dysfunction of the neuromuscular transmission can be found in vivo, and has been attributed to a combined effect of nerve degeneration and regeneration, with a small size of nerve terminals, and a conduction block in immature axons. Histological studies show (partially) denuded postsynaptic areas with a relatively intact structure of the folds, similar to some of the junctions shown herein.[170]

Disturbances in the apposition between pre- and postsynaptic neuromuscular junction structures have been demonstrated in mice after passive immunisation with patient anti-MuSK IgG.[171] These mice showed a myasthenic phenotype, substantiated by a decreased density of AChRs in fluorescence-labelling studies and a decrement of compound muscle action potentials in repetitive nerve stimulation electromyography. Unfortunately, no in vitro electrophysiological analysis was performed at the neuromuscular junctions.

In conclusion, our electrophysiological and histological data show not only postsynaptic defects at the neuromuscular junction of a MuSK MG patient, but also demonstrate presynaptic disturbances. It is highly likely that these neuromuscular synaptic deficits are caused by the circulating anti-MuSK antibodies and underlie the muscle weakness of the patient. Anti-MuSK antibodies may reduce the stability of the muscle-nerve contact, leading to a process of nerve retraction and regeneration.

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IX

Summary, discussion and future perspectives

Summary

Muscle-specific kinase (MuSK) antibodies were first described in 2001 by Hoch *et al*. [54] In this first cohort of selected patients with generalised myasthenia gravis (MG) without antibodies to the acetylcholinereceptor (AChR), 70% tested positive for these new anti-MuSK antibodies. In the Netherlands, we studied the epidemiology of generalised MG subtypes on a regional and national level. We used an area defined by postal codes in the Netherlands and patient registries in all hospitals within this region (Chapter 2). MuSK MG proved to be a rare disease with an incidence of 0.17 per million person-years, between January 1st 1990 and January 1st 2004. On January 1st 2004, the prevalence was 2.8 per million inhabitants.

We then conducted a nationwide study to include all traceable patients with generalised MG without antibodies to the AChR. This study yielded similar results, showing an incidence of MuSK MG of 0.10 per million per year and a prevalence of 1.9 per million inhabitants. Antibodies to the AChR were absent in 14.5% of all patients with generalised MG, of whom 22% were MuSK antibody positive. Thus, in the Netherlands approximately 3% of all generalised MG patients will have antibodies to MuSK. MuSK MG was characterised by a female predominance. The onset of symptoms in MuSK MG varied widely between 2 and 74.6 years.

In the clinical study (Chapter 3), we examined the distribution of weakness in generalised MG subtypes in more detail. We included the first 25 patients diagnosed with MuSK MG during the epidemiological study, and compared them to 50 randomly selected AChR MG controls matched for age at onset and sex. The first occurrence of symptoms in a period of 5 years after onset of the first MG symptom was investigated retrospectively based on a standardised history taking that was applied by a single investigator, and information from patients' charts. The same approach was used for all 41 patients with seronegative MG (SNMG). The first symptoms did not discriminate between the various MG subtypes. During the course of the study, a new cell-based technique became available, using recombinant MuSK or AChR transfected human embryonic kidney cells to test for autoantibodies to clustered or overexpressed antigens. Within the group of 41 SNMG patients defined by standard radioimmunoassay (RIA), this sensitive technique identified a further 10 patients with autoantibodies to AChR and another 10 who tested positive for MuSK autoantibodies. In the remaining 21 patients, now newly defined as seronegative, weakness was characterised by mild proximal limb-girdle distribution with frequent involvement of the extraocular muscles, with ptosis occurring more often than diplopia.

In patients with MuSK MG, weakness predominantly affected bulbar and neck muscles during the course of the disease. Among the symptoms pointing at bulbar muscle weakness, dysarthria and dysphagia were reported most often, followed by symptoms of facial weakness.

By contrast, chewing was relatively spared. Dyspnoea was reported more often by MuSK MG patients, and occasionally even as the first symptom. Respiratory support was needed within five years after the onset of symptoms in 28% of MuSK MG patients, as compared to only 10% of AChR MG patients. MuSK MG patients also showed an increased sensitivity of muscle fibers to acetylcholinesterase inhibitors leading to muscle twitching and cramps. The phenotype of patients with antibodies to overexpressed MuSK in the cell-based assay only was similar to that of patients who already tested positive for anti-MuSK antibodies in the conventional radioimmunoassay (RIA).

Among patients with MuSK MG, 22% had a second autoimmune disease and 35% had a first-degree relative with such a disorder, mainly affecting the thyroid gland. One of the best known genetic factors predisposing for autoimmune diseases is the HLA genotype. AChR MG without thymoma and the Lambert-Eaton myasthenic syndrome without small-cell lung cancer are both linked to the HLA-B8-DR3 haplotype. No tumour is associated with MuSK MG, so we hypothesised a similar association with HLA-B8-DR3 in this disease. However, in Chapter 4 we presented evidence that MuSK MG is strongly associated with HLA-DR14-DQ5. The same haplotype has also been linked to pemphigus vulgaris, an autoimmune disorder causing blistering of the skin. Interestingly, autoantibodies in both pemphigus and MuSK MG are mainly of the IgG4 subclass.

We studied the relation between clinical disease severity in MuSK MG patients and the titres of antigen-specific IgG subclasses (Chapter 5). For this purpose, we developed the Disease Severity Score (DSS), a new classification that can be used for retrospective MG studies. The score yielded semi-continuous variables that allowed regression analysis using a linear mixed effect model. In six patients who had been followed during 2.5 to 13.4 years and from whom 7 to 12 serum samples had been stored, we found a significant association between antigen-specific IgG4 titres and DSS on a group level (β coefficient 0.44, $p = 0.036$). No such association was found for IgG1 titres (β coefficient -0.32, $p = 0.13$). Total antigen-specific IgG was weakly associated with DSS. A positive correlation between anti-MuSK specific IgG4 and the DSS was found in 5 of the 6 patients.

In Chapter 6 we studied the presence of autoantibodies to ErbB receptors in patients with SNMG. ErbB receptors are transmembrane proteins in the neuromuscular junction and, as such, antigens potentially exposed at sites not hidden from the immune system. Animal studies had shown a crucial role for ErbB receptors in the development of the neuromuscular junction. However, we were unable to detect anti-ErbB antibodies in sera of 42 SNMG patients defined by RIA from the original cohort using fluorescence-activated cell sorting and dot blotting analysis on transfected human embryonic kidney cells.

Finally, two patients with MuSK MG are described in detail in Chapters 7 and 8. In the first case, maternal anti-MuSK antibodies caused a transient myasthenic syndrome in the neonate through cross-placental transport. Autoantibodies were again mainly of the IgG4

subclass in both mother and child. Symptoms in the neonate occurred with a slight delay of approximately 8 hours and disappeared in 6 days. In the second case report, a combination of pre- and postsynaptic defects was found in a 29-year old woman with MuSK MG. In vitro electrophysiological studies performed on an intercostal muscle 9 months after onset of symptoms revealed low miniature end-plate potentials and low levels of presynaptic acetylcholine release. Histology showed partially denervated postsynaptic areas and some degeneration of postsynaptic folds.

Discussion

Defining the exact prevalence of MG subtypes remains challenging. In the course of this study, radioimmunoassays became more widely available and the clinical characteristics of the MuSK MG subtype became better known. This facilitated the diagnosis of MuSK MG, increasing the calculated prevalence of the disease. Referral bias also complicates the comparison of epidemiological studies from various countries as is shown by the slightly higher proportion of MuSK antibodies in our nationwide study, as compared to regional figures (36% versus 22% of generalised AChR Ab- MG). Not all MG patients are referred to specialised neuromuscular clinics, especially when weakness is only mild. In Chapter 3 we show that this is more often the case in SNMG than in MuSK MG. During this study, newly developed cell-based assays became available, identifying patients who tested negative in the standard radioimmunoassay, with autoantibodies only to clustered AChR or overexpressed MuSK antigens. This technical progress will lead to a higher prevalence of MuSK MG at the expense of SNMG and complicates the comparison of studies, both over time and geographically.

The concept of a North-South gradient in the prevalence of MuSK MG originated from European and North American studies showing for example a prevalence of 0% in Norway, 22% in The Netherlands and 40 % in Italy. [157] However, it does not apply for Asian populations where 41% in Japan (northern latitude 35°) is higher than 26.7% in Korea (northern latitude 37°) and 2.5-4% in China (northern latitudes 32°–34°). [172] The female predominance in MuSK MG is consistent in all studied populations. Our observation of a relatively high proportion of black MuSK MG patients in the Netherlands has been confirmed in two American studies. In a cohort described in North-Carolina, 44% of MuSK MG patients were of African-American origin, although the percentage of black people in the general population is not mentioned. [110] Among AChR antibody negative MG patients in Alabama, MuSK antibodies were found in 50% of those from Afro-American origin. Among white AChR antibody negative MG patients, the proportion of MuSK antibodies was only 17%. [173]

In the ten years after the first publication of anti-MuSK antibodies, MuSK MG has emerged as a rare but distinct disease entity. The differences in clinical course, genetic susceptibility and pathogenesis between MuSK MG and AChR MG have been highlighted in this thesis. In 2004, the role of MuSK antibodies was still subject of debate because of the absence of AChR and MuSK deficiency in a thoroughly studied muscle biopsy of a MuSK MG patient. [126] However, the case of a mother and child described in Chapter 6 fits Koch's postulate originating from the field of microbiology, and adapted for the use in autoimmunity to determine the causality between antibody and disease. Here, the passive transfer of an autoimmune factor to a susceptible "host" results in induction of the disease. This has been confirmed in other cases of neonatal myasthenic syndromes in MuSK MG. [174-176] Final proof was published in 2008 by Cole *et al*. who showed that IgG derived from MuSK MG patients caused a neuromuscular transmission defect in mice with reduced postsynaptic AChR staining and a reduced apposition of the nerve terminal and the postsynaptic AChR. [171] The combination of pre- and postsynaptic abnormalities in MuSK MG was further substantiated by Mori *et al*., who showed that active immunisation in mice with the rat-MuSK ectodomain caused axonal sprouting, with or without nerve terminals and reduced density of AChR clusters. [177] Again, in vitro electrophysiology showed reduced MEPP amplitudes and a reduced quantal content. MuSK antibodies also impaired acetylcholinesterase function, which is consistent with clinical observations in patients of an increased sensitivity to acetylcholinesterase inhibitor treatment resulting in muscle cramps and fasciculations.
The crucial role of IgG4 MuSK antibodies described in Chapter 5 was proven recently by Klooster *et al*. in a passive transfer study using purified IgG4 and IgG1-3 fractions from MuSK MG patients. [178] In this study, purified IgG4, but not IgG1-3, caused myasthenic weakness in mice. In vitro electrophysiology revealed a reduction of postsynaptic acetylcholine sensitivity and exaggerated depression of presynaptic acetylcholine release which is similar to the human in vitro studies described in Chapter 9. The IgG4 antibody in general has interesting properties, *i.e.* the inability to activate complement and the possibility of Fab arm exchange which makes many IgG4 antibodies functionally monovalent. Mori *et al*. showed that these monovalent MuSK Fab arms are sufficient to inhibit agrin-induced AChR clustering and MuSK phosphorylation in C2C12 myotubes. [177]

Future perspectives

An IgG4 immune response is thought to be triggered by chronic antigen exposure. [129] This suggests that a constantly present environmental factor might be responsible for the initiation of the immune response. It would be interesting to study similarities between MuSK epitopes and various environmental bacterial or viral antigens as they might be

involved in the onset of MuSK MG. It is also possible that the humoral immune response to antigen exposure in general is different in MuSK MG patients. This can be investigated by studying IgG subclass response to other antigens such as tetanus toxin in MuSK MG patients, and compare them to AChR MG and healthy controls.

The apparent fluctuation in disease severity that has been associated to IgG4 titres should be studied in more detail. It is clear in MG that the autoantibody titre is not the most important factor to determine the severity of symptoms. Patients can be severely affected with very low autoantibody titres or vice versa. [37] Increasing evidence exists that the inflammatory properties of IgG depend on the type of glycosylation of the Fc moiety affecting the interaction with Fcγ receptors. [179] Fc-glycosylation can change over time and has been associated to fluctuation in disease severity in several autoimmune disorders like rheumatoid arthritis. [180] Recently, we were able to detect changes in total IgG Fc N-glycosylation in a cohort of LEMS and AChR MG patients. [181] It seems worthwhile and feasible to explore changes in total IgG glycosylation over time in longitudinal studies such as described in Chapter 5. It may be even more interesting to study the glycosylation of antigen-specific IgG although this is technically challenging.

The effect of MuSK antibodies on the neuromuscular transmission also needs to be clarified in more detail. MuSK is thought to act mainly in synapse development and maintenance, processes with a relatively low turnover. On the other hand, patients can respond to plasma exchange within one or more days suggesting a quickly reversible dysfunction. [110] This suggests that MuSK may also have a role in synaptic signal transmission itself, or that MuSK antibodies can block the function of the neuromuscular synapse without causing structural damage.

Recently, treatment with rituximab seemed to be effective in MuSK MG. [182] This monoclonal antibody binds to the CD20 antigen. This antigen is expressed on B-cells, but not on plasma cells. Currently it is believed that antibody-dependent cell mediated cytotoxicity is the major mechanism of action of rituximab induced B-cell depletion. It also leads to complement mediated cell lysis and apoptosis of B-cells, and may have a regulatory effect on the cell cycle. [183] Further studies should aim to determine whether this expensive but well tolerated therapy may even be the first choice in MuSK MG, and has a sustained effect. Early and effective treatment may prevent irreversible muscle atrophy later on in the course of the disease. Developing a good symptomatic treatment in MuSK MG is also important as AChE inhibitors are clearly not useful in MuSK MG. Ephedrine and salbutamol improve muscle weakness in the Dok7 congenital myasthenic syndrome. [184,185] Both substances act on β2-adrenergic receptors that have two major effects on muscle. The first is anabolic through activation of adenylate cyclase, induction of intracellular cyclic AMP, subsequent activation of protein kinase A, and the inhibition of proteolytic pathways. The second is through the phosphatidylinositol 3-kinase pathway leading to the activation of the serine-threonine

kinase Akt which then phosphorylates numerous intracellular targets. [186] Considering the close interaction between MuSK and Dok7, and the crucial role of phosphorylation in the neuromuscular synapse as described in the introduction of this thesis, the salbutamol-induced increase in kinase activity may partially compensate for reduced MuSK signalling caused by the autoantibodies. Defining a clinical parameter to study the effect of symptomatic medication is, however, challenging in a fluctuating and predominantly bulbar disease like MuSK MG.

New antigens involved in SNMG might be discovered in the near future. LRP4 may be a target although the pathogenicity of the antibodies described in MG patients until now remains to be proven. The same applies for other proteins that play a role in the neuromuscular junction and that are (partially) located extracellularly and therefore accessible to antibodies. Some are mutated in congenital myasthenic syndromes such as laminin β2 or agrin. [91,187] Autoantibodies to potassium channels or AChE have been detected in AChR MG, and may theoretically also be present in SNMG although their role remains to be clarified. [188,189]

One of the enigmas in the field of neuromuscular diseases in general,and no less in that of myasthenia gravis, is the distribution of clinical weakness *i.e.* the different response of various muscles to omnipresent autoantibodies or genetic mutations. This also applies for MuSK MG with its distinct oculobulbar and axial weakness. It is clear that some striated muscles are different from others. Many physiological and immunological aspects have been described. Extraocular muscles, often involved in MG, have more tonic contractile characteristics and a lower expression of complement inhibitory genes. The ocular motorneurons also have high firing frequencies. Such factors probably make their neuromuscular transmission particularly vulnerable to autoimmune pathology. [190] Differences of intrinsic MuSK expression in various rat muscles have also been reported. [191] Further research identifying the underlying cause of the vulnerability of some muscle and the resistance of others will probably benefit from technicological progress in the field of genetics, like the availability of cDNA or protein arrays. Nonetheless, careful study of the signs and symptoms of patients will be indispensable for the determination of the clinical relevance of these findings.

Nederlandstalige samenvatting

Inleiding

Myasthenia gravis (MG) is een ziekte die wordt gekenmerkt door in de tijd wisselende spierzwakte die op enig moment in het leven begint. In een gedetailleerde publicatie van Erb en Goldflam aan het eind van de 19e eeuw beschreven zij dat bij MG het hangen van de oogleden (ptosis) en oogbewegingsstoornissen die leiden tot dubbelzien (diplopie) vaak de eerste symptomen zijn. Men spreekt dan van oculaire MG. Dit kan gevolgd worden door zwakte van bulbaire spieren met moeite bij kauwen, slikken en spreken, en gaat vaak gepaard met uitbreiding van de spierzwakte naar nek, armen en benen. Dit wordt gegeneraliseerde MG genoemd. Kenmerkend zijn de sterke wisselingen van de spierzwakte en de toename daarvan aan het einde van de dag en na inspanning. In 1934 lokaliseerde Walker de oorzaak van MG in de spierzenuw overgang (neuromusculaire synaps) door een kortdurend effect van een middel dat de afbraak van acetylcholine remt. Acetylcholine wordt door de zenuw afgegeven en draagt het signaal over naar de spier door binding aan de postsynaptische acetylcholine receptor (AChR). In 1976 toonden Lindstrom *et al.* antilichamen tegen deze AChR aan bij 87% van in totaal 71 patiënten met gegeneraliseerde MG. Deze antilichamen worden door het eigen immuunsysteem gemaakt en zijn gericht tegen lichaamseigen eiwitten. Myasthenia gravis is daarmee een autoimmuunziekte. De autoantilichamen tegen de AChR zijn vooral van de IgG1- en IgG3-subklasse. Dit type antilichamen veroorzaakt de transmissiestoornis door beschadiging van de postsynaptische membraan via activering van complement, een versnelde afbraak van AChRs en mogelijk ook door het blokkeren van de acetylcholine bindingsplaatsen. De autoantilichamen kunnen worden aangetoond met een radioimmunoassay. Hierin binden de autoantilichamen aan radioactief gelabelde receptoren en vormen zo complexen die neerslaan. De hoeveelheid radioactiviteit wordt gemeten en is een maat voor de hoeveelheid antilichamen. Bij ongeveer 10 tot 15% van de patiënten met gegeneraliseerde MG konden in het bloed echter op deze manier geen antilichamen tegen de AChR worden gevonden. Deze patiënten werden geclassificeerd als seronegatieve MG (SNMG). In 2001 werden voor het eerst antilichamen tegen muscle-specific kinase (MuSK) beschreven bij 70% van een cohort geselecteerde patiënten met SNMG. Het doel van dit proefschrift was de klinische verschijnselen van deze nieuwe ziekte te onderzoeken en het onderliggende immunologische mechanisme te verhelderen.

Proefschrift

In **hoofdstuk 2** onderzochten we de epidemiologie van de verschillende vormen van gegeneraliseerde MG op zowel regionaal als landelijk niveau. Hiervoor definieerden we in Nederland een regio op basis van postcode en includeerden MG patiënten van alle ziekenhuizen binnen deze regio (Hoofdstuk 2). Het bleek dat MG met antilichamen tegen MuSK (MuSK

MG) een zeldzame aandoening was. De incidentie, dat wil zeggen het aantal patiënten dat de ziekte krijgt in een bepaalde periode, was 0,17 per miljoen persoonsjaren tussen 1 januari 1990 en 1 januari 2004. De prevalentie, of met andere woorden, alle patiënten die met de ziekte op een bepaald tijdstip in leven zijn, was 2,8 per miljoen inwoners op 1 januari 2004. Vervolgens deden we landelijk onderzoek om alle traceerbare patiënten met gegeneraliseerd MG zonder antilichamen tegen de AChR te includeren. De resultaten van dit onderzoek waren vergelijkbaar met die uit het regionale onderzoek. De incidentie van MuSK MG was 0,10 per miljoen persoonsjaren. De prevalentie was 1,9 per miljoen inwoners op 1 januari 2004. Van alle patiënten met gegeneraliseerde MG had 14,5% geen antilichamen tegen de AChR. Van deze seronegatieve patiënten had slechts 22% antilichamen tegen MuSK. Daarmee heeft ongeveer 3% van alle patiënten met gegeneraliseerde MG antilichamen tegen MuSK. Een van de kenmerken van MuSK MG was dat het vaker voorkomt bij vrouwen. De leeftijd waarop patiënten de eerste symptomen kregen varieerde sterk tussen 2 en 74 jaar.
In het klinisch onderzoek beschreven in **hoofdstuk 3**, onderzochten we de verdeling van de spierzwakte bij de drie vormen van gegeneraliseerde MG in detail. We includeerden de eerste 25 patiënten die vanuit het landelijk epidemiologisch onderzoek werden geïdentificeerd met MuSK MG en vergeleken hen met 50 patiënten met gegeneraliseerde MG en antilichamen tegen de AChR (AChR MG). Deze werden in de verhouding 2:1 willekeurig geselecteerd uit databases in Leiden en Groningen, waarbij ze werden gepaard wat betreft geslacht en de leeftijd waarop de eerste symptomen van de ziekte waren begonnen. Met behulp van gegevens uit de medische status en een hernieuwde anamnese met de patiënt werd het moment bepaald waarop symptomen voor het eerst waren opgetreden in de eerste 5 jaar na het begin van de ziekte. Dit werd ook gedaan bij patiënten met SNMG. Met behulp van alleen het symptoom dat het allereerst optrad kon geen onderscheid gemaakt worden tussen de verschillende vormen van MG. Gedurende de duur van dit onderzoek werd ook een andere techniek ontwikkeld om antilichamen tegen de AChR of MuSK aan te tonen. Deze techniek maakt gebruik van cellen die kunstmatig AChR of MuSK aan hun celoppervlakte tot expressie brengen. Na toevoegen van serum van patiënten kan door een kleuring het binden van antilichamen aan deze cellen worden aangetoond. Binnen de groep van 41 patiënten die volgens de gebruikelijke methode met een radioimmunoassay waren gediagnosticeerd als SNMG, bleken met deze nieuwe techniek bij 10 patiënten toch antilichamen tegen de AChR aantoonbaar. Nog eens 10 andere patiënten hadden antilichamen tegen MuSK. Bij de resterende 21 patiënten met SNMG bleek de zwakte voornamelijk in de proximale bekken- en schoudergordel spieren te zitten. Ook waren de externe oogspieren vaak betrokken wat meestal leidde tot ptosis (hangen van de oogleden) en in mindere mate tot diplopie (dubbelzien).
Patiënten met MuSK MG hadden met name zwakte van bulbaire spieren en nekspieren. Van de symptomen die wezen op zwakte van de bulbaire spieren werden dysarthrie (onduidelijke

spraak) en dysphagie (moeite met slikken) het vaakst genoemd, gevolgd door gelaatszwakte. Het kauwen was daarentegen relatief gespaard. Benauwdheid werd vaker als symptoom genoemd door patiënten met MuSK MG en bij een enkeling zelfs als het eerste symptoom van de ziekte. Binnen de eerste 5 jaar na het begin van de ziekte kwam 28% van de MuSK MG patiënten op de IC terecht voor een periode met kunstmatige beademing in vergelijking met maar 10% van de patiënten met AChR MG. Bij patiënten met AChR MG gaf medicatie die de afbraak remt van acetylcholine door remming van het enzym acetylcholinesterase vaak een verbetering van de spierzwakte. De spieren van patiënten met MuSK MG waren daarentegen vaker overgevoelig voor deze medicatie wat bleek uit het spontaan samentrekken van spiervezels zonder dat de spierzwakte verbeterde. De verschijnselen van de 10 patiënten bij wie de antilichamen tegen MuSK alleen aantoonbaar waren met behulp van de nieuwe cel techniek waren identiek aan die van de 25 MuSK MG patiënten bij wie de antilichamen met behulp van de gebruikelijke radioimmunoassay gevonden waren.

In **hoofdstuk** 4 wordt beschreven dat 22% van de patiënten met MuSK MG een tweede autoimmuunziekte heeft en 35% een eerstegraads familielid met een dergelijk aandoening. Een van de bekendste genetische factoren die de gevoeligheid voor het krijgen van autoimmuunziekten bepaalt zijn de HLA genen. Twee vormen van myasthenie, namelijk die met antilichamen tegen de AChR en die met antilichamen tegen een presynaptisch calcium kanaal (het Lambert-Eaton myastheen syndroom), zijn geassocieerd met HLA-B8-DR3 indien de patiënten geen tumor hebben (geen thymoom bij AChR MG of geen kleincellig longcarcinoom bij LEMS). Aangezien bij MuSK MG geen tumoren zijn beschreven veronderstelden we dat HLA-B8-DR3 ook bij deze patiënten vaker gevonden zou worden. Zoals beschreven in hoofdstuk 4 bleek echter dat MuSK MG geassocieerd is met een ander type HLA, namelijk HLA-DR14-DQ5. Dit type komt ook vaker voor bij patiënten met pemphigus vulgaris. Dit is een autoimmuunziekte van de huid die leidt tot blaarvorming. Belangwekkend is dat de autoantilichamen bij zowel pemphigus als MuSK MG van een bepaald type zijn, de IgG4 subklasse.

In **hoofdstuk 5** wordt de relatie tussen de ernst van de symptomen en de hoeveelheid antilichamen tegen MuSK van verschillende IgG subklassen beschreven. We ontwikkelden een score systeem dat het mogelijk maakte retrospectief de ernst van de symptomen te bepalen. Deze score leverde een variabele op die gebruikt kon worden voor statistische analyse met behulp van regressie analyse in een lineair gemengd effect model. We konden 6 patiënten met MuSK MG includeren die gedurende 2,5 tot 13,4 jaar waren gevolgd en van wie er bij ieder tussen de 7 en 12 bloedmonsters waren bewaard. Op groepsniveau vonden we een verband tussen de ziekte ernst en de hoogte van de IgG4 antilichamen tegen MuSK, maar niet de hoogte van IgG1 antilichamen of van de totale hoeveelheid IgG tegen MuSK. Bij analyse van de patiënten afzonderlijk bleek er een positieve relatie tussen de hoogte van de IgG4 antilichamen tegen MuSK en de ernst van de symptomen bij 5 van de 6 patiënten.

In **hoofdstuk 6** onderzochten we of er bij patiënten met SNMG antilichamen waren tegen een ander eiwit, de ErbB receptor. ErbB receptoren zijn eiwitten die in de spiercelmembraan van de neuromusculaire synaps tot expressie komen. Ze kunnen daarom mogelijk door het afweersysteem worden gezien en dienen als antigeen voor een autoimmuunziekte. In dierexperimenteel onderzoek is ook een belangrijke rol van deze eiwitten bij de aanleg van de neuromusculaire synaps beschreven. Met behulp van cellen die ErbB receptoren kunstmatig tot expressie brengen en twee technieken om eventueel gebonden antilichamen zichtbaar te maken, konden we echter geen antilichamen tegen ErbB receptoren vinden in het bloed van 42 patiënten met SNMG.
Tenslotte wordt in de hoofdstukken 7 en 8 de ziektegeschiedenis van enkele patiënten beschreven. Bij de eerste casus in **hoofdstuk 7** veroorzaakten antilichamen tegen MuSK die aanwezig zijn in het bloed van de moeder een tijdelijke myasthene spierzwakte bij haar pasgeboren kind. Ook deze antilichamen waren vooral van de IgG4 subklasse. De verschijnselen bij het kind begonnen ongeveer 8 uur na de geboorte en verdwenen op de zesde dag. Een dergelijke overdracht van symptomen door antilichamen is belangrijk omdat het één van de bewijzen vormt dat deze antilichamen ook daadwerkelijk ziekmakend zijn.
De tweede casus in **hoofdstuk 8** beschrijft een vrouwelijke patiënt met MuSK MG bij wie een spierbiopt met daarin neuromusculaire synapsen is onderzocht. Dit was gedaan in de tijd dat antilichamen tegen MuSK nog niet waren ontdekt met het doel de diagnose MG te bevestigen. Elektrofysiologisch onderzoek van de spierzenuw overgang liet zien dat de signaaloverdracht was verminderd. Deze verstoring bevond zich zowel aan de kant van de zenuw (presynaptisch, er werd minder acetylcholine afgegeven) als aan de kant van de spier (postsynaptisch, het door de zenuw afgegeven acetylcholine genereerde een te lage elektrische potentiaal). Dit is anders dan bij AChR MG waar het probleem postsynaptisch is door een tekort aan receptoren. Licht- en elektronenmicroscopisch onderzoek liet zien dat delen van de postsynaptische spiermembraan geen tegenoverliggende zenuw meer hadden en dat de plooiing van deze spiermembraan was verminderd.
Samenvattend is in dit proefschrift beschreven dan MuSK MG een zeldzame maar duidelijk te onderscheiden ziekte is. Zowel de klinische verschijnselen, de genetische aanleg om de ziekte te krijgen, het type antilichaam waarmee het immuunsysteem de ziekte veroorzaakt en manier waarop de antilichamen de signaaloverdracht in de spierzenuw overgang verstoren, zijn duidelijk anders dan bij de veel langer bekende aandoening AChR MG.

X

Appendices

List of abbreviations

AAEM	American Association of Electrodiagnostic Medicine
Ab +/-	antibody positive / negative
ACh	acetylcholine
AChE	acetylcholinesterase
AChEI	acetylcholinesterase inhibitor
AChR	acetylcholine receptor
AChR MG	myasthenia gravis with autoantibodies to AChR
ATP	adenosine triphosphate
AU	arbitrary units
CI	confidence interval
CK	creatine kinase
CMAP	compound muscle action potential
ColQ	collagen Q
CV	coefficient of variation
DHPR	dihydropyridine receptor
DSS	disease severity score
EGFP	enhanced green fluorescent protein
EMG	electromyography
EPP	endplate potential
FACS	fluorescence-activated cell sorting
GMG	generalised myasthenia gravis
HEK	human embryonic kidney
HLA	human leukocyte antigen
Ig	immunoglobulin
IV	intravenous
IVIG	intravenous human immunoglobulin
kDa	kilo Dalton
LEMS	Lambert-Eaton myasthenic syndrome
LRP4	low-density lipoprotein receptor-related protein
MASC	myotube associated specificity component
MEPP	miniature endplate potential
MFI	mean fluorescence intensity
MG	myasthenia gravis
MGFA	Myasthenia Gravis Foundation of America
MIP	maximal inspiratory pressure
MRI	magnetic resonance imaging

MuSK	muscle-specific kinase
MuSK MG	myasthenia gravis with autoantibodies to MuSK
NHS	normal human serum
NMJ	neuromuscular junction
OD	optical density
OMG	ocular myasthenia gravis
OR	odds ratio
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	plasma exchange
PEI	polyethylenimine
PV	pemphigus vulgaris
RIA	radioimmunoassay
RNA	ribonucleic acid
RNS	repetitive nerve stimulation
RT	room temperature
SFEMG	single-fiber electromyography
SNMG	seronegative MG – MG without autoantibodies to AChR or MuSK
Tid1	tumorous imaginal disc protein
TX	thymectomy
VGCC	voltage-gated calcium channel

List of publications

P.K. Gregersen, R. Kosoy, A.T. Lee, J. Lamb, J. Sussman, D. McKee, K.R. Simpfendorfer, R. Pirskanen-Matell, F. Pieh, Q. Pan-Hammarstrom, J.J.G.M.Verschuuren, M.J. Titulaer, **E.H. Niks**, A. Marx, P. Ströbel, B.Tackenberg, M. Püetz, A.Maniaol, A. Elsais, C. Tallaksen, H.F. Harbo, B.A. Lie, S. Raychaudhuri, P.I.W. de Bakker, A. Melms, H.J. Garchon, N. Willcox, L. Hammarstrom, M.F. Seldin. Risk for Myasthenia Gravis maps to 151Pro→Ala change in TNIP1 and to HLA-B*08. Ann Neurol 2012. Epub 2012 Oct 10.

E.H. Niks, M.H. De Baets, J.J.G.M. Verschuuren. IgG4-related disease. N Engl J Med. 2012; 366(17):1644-5.

R. Klooster, J.J. Plomp, M.G. Huijbers, **E.H. Niks**, K.R. Straasheijm, F.J. Detmers, P.W. Hermans, K. Sleijpen, A, Verrips, M. Losen, P. Martinez-Martinez, M.H. De Baets, S.M. van der Maarel, J.J.G.M. Verschuuren. Muscle-specific kinase myasthenia gravis IgG4 autoantibodies cause severe neuromuscular junction dysfunction in mice. Brain 2012; 135:1081-1101. Epub 2012 Mar 6.

I.A. Ketelslegers, C.E. Catsman-Berrevoets, R.F. Neuteboom, M. Boon, K.G. van Dijk, M.J Eikelenboom, R.H. Gooskens, **E.H. Niks**, W.C. Overweg-Plandsoen, E.A. Peeters, C.M. Peeters-Scholte, B.T. Poll-The, J.F. de Rijk-van Andel, J.P. Samijn, I.N. Snoeck, H. Stroink, R.J. Vermeulen, A. Verrips, J.S. Vles, M.A. Willemsen, R. Rodrigues Pereira, R.Q. Hintzen. Incidence of acquired demyelinating syndromes of the CNS in Dutch children: a nationwide study. J Neurol. 2012; 259(9):1929-35. Epub 2012 Feb 17.

K. Vrolix, **E.H. Niks**, R. Le Panse, M.M. van Ostaijen-Ten Dam, A.H. Muris, C.M. Jol-van der Zijde, M.J. van Tol, M. Losen, P.C. Molenaar, E.J. van Zoelen, S. Berrih-Aknin, M.H. De Baets, J.J. Verschuuren, P. Martínez-Martínez. Reduced thymic expression of ErbB receptors without auto-antibodies against synaptic ErbB in myasthenia gravis. J Neuroimmunol 2011; 232(1-2):158-165. Epub 2010 Dec 18.

M.H. Selman, **E.H. Niks**, M.J. Titulaer, J.J.G.M. Verschuuren, M. Wuhrer, A.M. Deelder. IgG Fc N-Glycosylation Changes in Lambert-Eaton Myasthenic Syndrome and Myasthenia Gravis. J Proteome Res. 2011; 10(1):143-152. Epub 2010 Jul 30.

E.H. Niks, J.B.M. Kuks, J.H.J. Wokke, H. Veldman, E. Bakker, J.J.G.M. Verschuuren, J.J. Plomp. Pre- and postsynaptic neuromuscular junction abnormalities in MuSK myasthenia. Muscle Nerve 2010; 42(2): 283-288.

A.M.G. Sas, **E.H. Niks**, M.H. Lequin, C.E. Catsman-Berrevoets, M.C.Y. de Wit. Herpes simplex virus type-1 encephalitis and occipital ischemic stroke. Pediatr Neurol. 2009; 41(4): 294-296.

W.P. ter Beek. Martínez-Martínez, M. Losen, M.H. de Baets, A.R. Wintzen, J.J.G.M. Verschuuren, **E.H. Niks**, S.G. van Duinen, A. Vincent, P.C. Molenaar. The Effect of Plasma From Muscle-Specific Tyrosine Kinase Myasthenia Patients on Regenerating Endplates. Am J Pathol 2009; 175(4): 1536-1544. Epub 2009 Sep 10.

F.M. Cox, J.J.G.M. Verschuuren, B.M. Verbist, **E.H. Niks**, A.R. Wintzen, U.A. Badrising. Detecting dysphagia in inclusion body myositis. J Neurol 2009; 256(12): 2009-2013. Epub 2009 Jul 15.

E.H. Niks, Y. van Leeuwen, M.I. Leite, F.W. Dekker, A.R. Wintzen, P.W. Wirtz, A. Vincent, M.J.D. van Tol, C.M. Jol-van der Zijde, J.J.G.M. Verschuuren. Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1. J Neuroimmunol 2008; 195(1-2): 151-156. Epub 2008 Apr 1.

E.H. Niks, A. Verrips, B.A. Semmekrot, M.J.J. Prick, A. Vincent, M.J.D. van Tol, C.M. Jol-van der Zijde, J.J.G.M. Verschuuren. A transient neonatal myasthenic syndrome with anti-MuSK antibodies. Neurology 2008; 70(14): 1215-1216.

M.I. Leite, M. Jones, P. Strobel, A. Marx, R. Gold, **E.H. Niks**, J.J.G.M.Verschuuren, S. Berrih-Aknin, F. Scaravilli, A. Canelhas, B.P. Morgan, A. Vincent, N. Willcox. Myasthenia Gravis Thymus: Complement Vulnerability of Epithelial and Myoid Cells, Complement Attack on Them, and Correlations with Autoantibody Status. Am J Pathol 2007; 171(3): 893-905.

E.H. Niks, J.B.M. Kuks, J.J.G.M. Verschuuren. Epidemiology of myasthenia gravis with anti-muscle specific kinase antibodies in The Netherlands. J Neurol Neurosurg Psychiatry 2007; 78(4): 417-418. Epub 2006 Oct 20.

E.H. Niks, J.B.M. Kuks, B.O. Roep, G.W. Haasnoot, W. Verduijn, B.E.P.B. Ballieux, M.H. De Baets, A. Vincent, J.J.G.M. Verschuuren. Strong association of MuSK antibody-positive myasthenia gravis and HLA-DR14-DQ5. Neurology 2006; 66(11): 1772-1774.

M.I. Leite, P. Strobel, M. Jones, K. Micklem, R. Moritz, R. Gold, **E.H. Niks**, S. Berrih-Aknin, F. Scaravilli, A. Canelhas, A. Marx, J. Newsom-Davis, N. Willcox, A.Vincent. Fewer thymic changes in MuSK antibody-positive than in MuSK antibody-negative MG. Ann Neurol 2005; 57(3): 444-8.

E.H. Niks, J.B.M. Kuks, M.H. De Baets, J.J.G.M. Verschuuren. Myasthenia gravis door antilichamen tegen het Muscle Specific Kinase.
Tijdschrift voor Neurologie en Neurochirurgie 2004; 105(6): 253-258.

E.H. Niks, U.A. Badrising, J.J.G.M. Verschuuren, J.G. van Dijk. Decremental response of the nasalis and hypothenar muscles in myasthenia gravis. Muscle Nerve 2003; 28(2): 236-238.

E.H. Niks, J.J.G.M. Verschuuren. Een patiënt met een wisselende, asymmetrische ptosis. Nederlands Tijdschrift voor Neurologie 2002; 3: 212-214.

Curriculum Vitae

Erik Harmen Niks was born on May 7, 1972 in Veenendaal, the Netherlands. He attended secondary school at the Christelijk Lyceum Veenendaal, and graduated with an average final mark of 9.5 in 1990. In 1991 he started his medical training at the University of Utrecht, and graduated cum laude for the propaedeutic diploma the next year. After obtaining his physician's degree in 1999 at the University Medical Center in Utrecht, he became a resident in pediatrics at the Leiden University Medical Center (LUMC, Prof. Dr. J.M. Wit). In 2000, he started as a resident in neurology at the LUMC (Prof. Dr. R.A.C. Roos). Part of his training was at the Haga Hospital in The Hague (Dr. S.F.T.M. de Bruijn). He started his PhD study in 2002 (Prof. Dr. A.R. Wintzen and Prof. Dr. J.J.G.M. Verschuuren). In 2008, he was trained in pediatric neurology at the Erasmus Medical Center in Rotterdam (Prof. Dr. W.F. Arts). Later that year, he joined the neurology staff at the LUMC. He registered as pediatric neurologist in 2009. He received a Prinses Beatrix Fonds fellowship to specialise in myology. This fellowship, completed in 2012, included the work as honorary clinical fellow at the Dubowitz Neuromuscular Centre in London (Prof. F. Muntoni). He is currently involved in the care of children with neuromuscular disorders, and in the translational research of Duchenne muscular dystrophy at the LUMC.

During secondary school he received piano lessons from Ton Hartsuiker, head of the Utrecht Conservatory of Music. In 1988 he made his solo debut in Mozart's eleventh piano concerto. In that same year, he commenced the preparatory class at the Utrecht School of the Arts. He received prizes in various youth music contests, including the Prinses Christina competition and the Steinway competition. In 1990 he started his master study piano with the Dutch pianist Herman Uhlhorn. From 1993 until his graduation as soloist in 1996, he studied with the Norwegian pianist Håkon Austbö. Until 1999 he performed frequently with the Rosa Ensemble, an ensemble specialising in contemporary music and theatre productions. In 2006 and 2007 he played the Grieg piano concerto, accompanied by the I Medici orchestra, consisting of medical professionals. Currently, he performs with his wife, the viola player Karen de Wit. They live in Leiden and have two children, Geert and Elin.

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References

1. Osserman K. Myasthenia gravis. New York: Grune & Stratton; 1958.
2. Erb W. Zur Casuistik der bulbären Lähmungen. 3 Ueber einen neuen, wahrscheinlich bulbären Symptomencomplex. Archiv für Psychiatrie und Nervenkrankheiten 1879;9:336-350.
3. Goldflam S. Ueber einen scheinbar heilbaren bulbär paralytischen Symptomencomplex mit Beteiligung der Extremitäten. Deutsches Zeitschrift für Nervenheilkunde 1893;4:213-352.
4. Walker MB. Treatment of myasthenia gravis with physostigmine. Lancet 1934;223 (5779):1200-1201.
5. Dale HH, Feldberg W. Chemical transmission at motor nerve endings in voluntary muscle. J Physiol (Lond) 1934;81:39P.
6. Coërs C, Desmedt JE. Mise en évidence d'une malformation caractéristique de la jonction neuromusculaire dans la myasthénie. Acta Neurol Psychiatr Belg 1959;59(5):539-561.
7. Simpson JA. Myasthenia gravis: a new hypothesis. Scot Med J 1960;5(10):419-436.
8. Patrick J, Lindstrom J. Autoimmune response to acetylcholine receptor. Science 1973;180(88):871-872.
9. Lindstrom JM, Seybold ME, Lennon VA, Whittingham S, Duane DD. Antibody to acetylcholine receptor in myasthenia gravis. Prevalence, clinical correlates, and diagnostic value. Neurology 1976;26(11):1054-1059.
10. Besinger UA, Toyka KV, Homberg M, Heininger K, Hohlfeld R, Fateh-Moghadam A. Myasthenia gravis: long-term correlation of binding and bungarotoxin blocking antibodies against acetylcholine receptors with changes in disease severity. Neurology 1983;33(10):1316-1321.
11. Drachman DB, Angus CW, Adams RN, Michelson JD, Hoffman GJ. Myasthenic antibodies cross-link acetylcholine receptors to accelerate degradation. N Engl J Med 1978;298(20):1116-1122.
12. Lennon VA, Seybold ME, Lindstrom JM, Cochrane C, Ulevitch R. Role of complement in the pathogenesis of experimental autoimmune myasthenia gravis. J Exp Med 1978;147(4):973-983.
13. Engel AG, Lambert EH, Howard FM. Immune complexes (IgG and C3) at the motor end-plate in myasthenia gravis: ultrastructural and light microscopic localization and electrophysiologic correlations. Mayo Clin Proc 1977;52(5):267-280.
14. Sahashi K, Engel AG, Lambert EH, Howard FM, Jr. Ultrastructural localization of the terminal and lytic ninth complement component (C9) at the motor end-plate in myasthenia gravis. J Neuropathol Exp Neurol 1980;39(2):160-172.

15. Rodgaard A, Nielsen FC, Djurup R, Somnier F, Gammeltoft S. Acetylcholine receptor antibody in myasthenia gravis: predominance of IgG subclasses 1 and 3. Clin Exp Immunol 1987;67(1):82-88.
16. Hunt PJ, Marshall SE, Weetman AP et al. Histocompatibility leucocyte antigens and closely linked immunomodulatory genes in autoimmune thyroid disease. Clin Endocrinol (Oxf) 2001;55(4):491-499.
17. Alper CA, Fleischnick E, Awdeh Z, Katz AJ, Yunis EJ. Extended major histocompatibility complex haplotypes in patients with gluten-sensitive enteropathy. J Clin Invest 1987;79(1):251-256.
18. Wirtz PW, Willcox N, van der Slik AR et al. HLA and smoking in prediction and prognosis of small cell lung cancer in autoimmune Lambert-Eaton myasthenic syndrome. J Neuroimmunol 2005;159(1-2):230-237.
19. Price P, Witt C, Allcock R et al. The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological diseases. Immunol Rev 1999;167:257-274.
20. Giraud M, Beaurain G, Yamamoto AM et al. Linkage of HLA to myasthenia gravis and genetic heterogeneity depending on anti-titin antibodies. Neurology 2001;57(9):1555-1560.
21. Janer M, Cowland A, Picard J et al. A susceptibility region for myasthenia gravis extending into the HLA-class I sector telomeric to HLA-C. Hum Immunol 1999;60(9):909-917.
22. Kuks JBM, Lems SPM, Oosterhuis HJGH. HLA type is not indicative for the effect of thymectomy in myasthenia gravis. J Neuroimmunol 1992;36(2-3):217-224.
23. Compston DA, Vincent A, Newsom-Davis J, Batchelor JR. Clinical, pathological, HLA antigen and immunological evidence for disease heterogeneity in myasthenia gravis. Brain 1980;103(3):579-601.
24. Feltkamp TE, van den Berg-Loonen PM, Nijenhuis LE et al. Myasthenia gravis, autoantibodies, and HL-A antigens. Br Med J 1974;1(899):131-133.
25. Oosterhuis HJ, Feltkamp TE, van Rossum AL, van den Berg-Loonen PM, Nijenhuis LE. HL-A antigens, autoantibody production, and associated diseases in thymoma patients, with and without myasthenia gravis. Ann N Y Acad Sci 1976;274:468-474.
26. Antozzi C, Gemma M, Regi B et al. A short plasma exchange protocol is effective in severe myasthenia gravis. J Neurol 1991;238(2):103-107.
27. Evoli A, Bartoccioni E, Batocchi AP, Scuderi F, Tonali P. Anti-AChR-negative myasthenia gravis: clinical and immunological features. Clin Invest Med 1989;12(2):104-109.

28. Garlepp MJ, Dawkins RL, Christiansen FT et al. Autoimmunity in ocular and generalised myasthenia gravis. J Neuroimmunol 1981;1(3):325-332.
29. Lefvert AK, Bergstrom K, Matell G, Osterman PO, Pirskanen R. Determination of acetylcholine receptor antibody in myasthenia gravis: clinical usefulness and pathogenetic implications. J Neurol Neurosurg Psychiatry 1978;41(5):394-403.
30. Lennon VA, Howard FM. Serologic diagnosis of myasthenia gravis. In: Nakamura R, editor. Clinical laboratory molecular analyses. Orlando, FL: Grune & Stratton; 1985:29-44.
31. Limburg PC, The TH, Hummel-Tappel E, Oosterhuis HJ. Anti-acetylcholine receptor antibodies in myasthenia gravis. Part 1. Relation to clinical parameters in 250 patients. J Neurol Sci 1983;58(3):357-370.
32. Morel E, Raimond F, Goulon-Goeau C et al. Le dosage des anticorps anti-recepteurs de l'acetylcholine dans la myasthenie: etude de 329 serums. Nouv Presse Med 1982;11(24):1849-1854.
33. Newsom-Davis J, Willcox N, Schluep M et al. Immunological heterogeneity and cellular mechanisms in myasthenia gravis. Ann N Y Acad Sci 1987;505:12-26.
34. Soliven BC, Lange DJ, Penn AS et al. Seronegative myasthenia gravis. Neurology 1988;38(4):514-517.
35. Tindall RS. Humoral immunity in myasthenia gravis: biochemical characterization of acquired antireceptor antibodies and clinical correlations. Ann Neurol 1981;10(5):437-447.
36. Sanders DB, Andrews PI, Howard JF, Massey JM. Seronegative myasthenia gravis. Neurology 1997;48(Suppl 5):S40-S45.
37. Oosterhuis HJ, Limburg PC, Hummel-Tappel E, The TH. Anti-acetylcholine receptor antibodies in myasthenia gravis. Part 2. Clinical and serological follow-up of individual patients. J Neurol Sci 1983;58(3):371-385.
38. Birmanns B, Brenner T, Abramsky O, Steiner I. Seronegative myasthenia gravis: clinical features, response to therapy and synthesis of acetylcholine receptor antibodies in vitro. J Neurol Sci 1991;102(2):184-189.
39. Andrews PI, Massey JM, Sanders DB. Acetylcholine receptor antibodies in juvenile myasthenia gravis. Neurology 1993;43(5):977-982.
40. Oger J, Kaufman R, Berry K. Acetylcholine receptor antibodies in myasthenia gravis: use of a qualitative assay for diagnostic purposes. Can J Neurol Sci 1987;14(3):297-302.
41. Marengo TS, Harrison R, Lunt GG, Behan PO. Rat or human acetylcholine-receptor antigen for investigation of myasthenia gravis? Lancet 1979;1(8113):442.

42. Vincent A, Newsom-Davis J. Acetylcholine receptor antibody as a diagnostic test for myasthenia gravis: results in 153 validated cases and 2967 diagnostic assays. J Neurol Neurosurg Psychiatry 1985;48(12):1246-1252.
43. Beeson D, Jacobson L, Newsom-Davis J, Vincent A. A transfected human muscle cell line expressing the adult subtype of the human muscle acetylcholine receptor for diagnostic assays in myasthenia gravis. Neurology 1996;47(6):1552-1555.
44. Leite MI, Jacob S, Viegas S et al. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. Brain 2008;131(Pt 7):1940-1952.
45. Burke G, Cossins J, Maxwell S et al. Rapsyn mutations in hereditary myasthenia: Distinct early- and late-onset phenotypes. Neurology 2003;61(6):826-828.
46. Muller JS, Herczegfalvi A, Vilchez JJ et al. Phenotypical spectrum of DOK7 mutations in congenital myasthenic syndromes. Brain 2007;130(Pt 6):1497-1506.
47. Grob D, Simpson D, Mitsumoto H et al. Treatment of myasthenia gravis by immunoadsorption of plasma. Neurology 1995;45(2):338-344.
48. Miller RG, Milner-Brown HS, Dau PC. Antibody-negative acquired myasthenia gravis: successful therapy with plasma exchange. Muscle Nerve 1981;4(3):255.
49. Melber D. Maternal-fetal transmission of myasthenia gravis with acetylcholine-receptor antibody. N Engl J Med 1988;318(15):996.
50. Mier AK, Havard CW. Diaphragmatic myasthenia in mother and child. Postgrad Med J 1985;61(718):725-727.
51. Burges J, Vincent A, Molenaar PC, Newsom-Davis J, Peers C, Wray D. Passive transfer of seronegative myasthenia gravis to mice. Muscle Nerve 1994;17(12):1393-1400.
52. Mossman S, Vincent A, Newsom-Davis J. Myasthenia gravis without acetylcholine-receptor antibody: a distinct disease entity. Lancet 1986;1(8473):116-119.
53. Blaes F, Beeson D, Plested P, Lang B, Vincent A. IgG from "seronegative" myasthenia gravis patients binds to a muscle cell line, TE671, but not to human acetylcholine receptor. Ann Neurol 2000;47(4):504-510.
54. Hoch W, McConville J, Helms S, Newsom-Davis J, Melms A, Vincent A. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. Nat Med 2001;7(3):365-368.
55. Scuderi F, Marino M, Colonna L et al. Anti-p110 autoantibodies identify a subtype of "seronegative" myasthenia gravis with prominent oculobulbar involvement. Lab Invest 2002;82(9):1139-1146.
56. Evoli A, Tonali PA, Padua L et al. Clinical correlates with anti-MuSK antibodies in generalized seronegative myasthenia gravis. Brain 2003;126(10):2304-2311.
57. Evoli A, Batocchi AP, Lo MM et al. Clinical heterogeneity of seronegative myasthenia gravis. Neuromuscular Disorders 1996;6(3):155-161.

58. Reist NE, Werle MJ, McMahan UJ. Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. Neuron 1992;8(5):865-868.
59. Jennings CG, Dyer SM, Burden SJ. Muscle-specific trk-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases. Proc Natl Acad Sci USA 1993;90(7):2895-2899.
60. Hopf C, Hoch W. Dimerization of the muscle-specific kinase induces tyrosine phosphorylation of acetylcholine receptors and their aggregation on the surface of myotubes. J Biol Chem 1998;273(11):6467-6473.
61. Valenzuela DM, Stitt TN, DiStefano PS et al. Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. Neuron 1995;15(3):573-584.
62. Till J, Becerra M, Watty A et al. Crystal structure of the MuSK tyrosine kinase: insights into receptor autoregulation. Structure 2002;10(9):1187-1196.
63. Johnson LN, Noble ME, Owen DJ. Active and inactive protein kinases: structural basis for regulation. Cell 1996;85(2):149-158.
64. Zhu D, Yang Z, Luo Z, Luo S, Xiong WC, Mei L. Muscle-specific receptor tyrosine kinase endocytosis in acetylcholine receptor clustering in response to agrin. J Neurosci 2008;28(7):1688-1696.
65. DeChiara TM, Bowen DC, Valenzuela DM et al. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 1996;85(4):501-512.
66. Kong XC, Barzaghi P, Ruegg MA. Inhibition of synapse assembly in mammalian muscle in vivo by RNA interference. EMBO Rep 2004;5(2):183-188.
67. Gautam M, Noakes PG, Moscoso L et al. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell 1996;85(4):525-535.
68. Mittaud P, Camilleri AA, Willmann R, Erb-Vogtli S, Burden SJ, Fuhrer C. A single pulse of agrin triggers a pathway that acts to cluster acetylcholine receptors. Mol Cell Biol 2004;24(18):7841-7854.
69. Stiegler AL, Burden SJ, Hubbard SR. Crystal structure of the agrin-responsive immunoglobulin-like domains 1 and 2 of the receptor tyrosine kinase MuSK. J Mol Biol 2006;364(3):424-433.
70. Glass DJ, Bowen DC, Stitt TN et al. Agrin acts via a MuSK receptor complex. Cell 1996;85(4):513-523.
71. Glass DJ, Apel ED, Shah S et al. Kinase domain of the muscle-specific receptor tyrosine kinase (MuSK) is sufficient for phosphorylation but not clustering of acetylcholine receptors: required role for the MuSK ectodomain? Proc Natl Acad Sci USA 1997;94(16):8848-8853.

72. Weatherbee SD, Anderson KV, Niswander LA. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development 2006;133(24):4993-5000.
73. Kim N, Stiegler AL, Cameron TO et al. Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell 2008;135(2):334-342.
74. Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L. LRP4 serves as a coreceptor of agrin. Neuron 2008;60(2):285-297.
75. Gautam M, Noakes PG, Mudd J et al. Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. Nature 1995;377(6546):232-236.
76. Borges LS, Yechikhov S, Lee YI et al. Identification of a motif in the acetylcholine receptor beta subunit whose phosphorylation regulates rapsyn association and postsynaptic receptor localization. J Neurosci 2008;28(45):11468-11476.
77. Okada K, Inoue A, Okada M et al. The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science 2006;312(5781):1802-1805.
78. Bergamin E, Hallock PT, Burden SJ, Hubbard SR. The cytoplasmic adaptor protein Dok7 activates the receptor tyrosine kinase MuSK via dimerization. Mol Cell 2010;39(1):100-109.
79. Linnoila J, Wang Y, Yao Y, Wang ZZ. A mammalian homolog of Drosophila tumorous imaginal discs, Tid1, mediates agrin signaling at the neuromuscular junction. Neuron 2008;60(4):625-641.
80. Cartaud A, Strochlic L, Guerra M et al. MuSK is required for anchoring acetylcholinesterase at the neuromuscular junction. J Cell Biol 2004;165(4):505-515.
81. Lu Z, Je HS, Young P, Gross J, Lu B, Feng G. Regulation of synaptic growth and maturation by a synapse-associated E3 ubiquitin ligase at the neuromuscular junction. J Cell Biol 2007;177(6):1077-1089.
82. Hezel M, de Groat WC, Galbiati F. Caveolin-3 promotes nicotinic acetylcholine receptor clustering and regulates neuromuscular junction activity. Mol Biol Cell 2009;21(2):302-310.
83. Ghazanfari N, Fernandez KJ, Murata Y et al. Muscle specific kinase: organiser of synaptic membrane domains. Int J Biochem Cell Biol 2011;43(3):295-298.
84. Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee KF. Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature 2001;410(6832):1057-1064.
85. Yang X, Arber S, William C et al. Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron 2001;30(2):399-410.

86. Kim N, Burden SJ. MuSK controls where motor axons grow and form synapses. Nat Neurosci 2008;11(1):19-27.
87. Chen F, Liu Y, Sugiura Y, Allen PD, Gregg RG, Lin W. Neuromuscular synaptic patterning requires the function of skeletal muscle dihydropyridine receptors. Nat Neurosci 2011;14(5):570-577.
88. Beeson D, Higuchi O, Palace J et al. Dok-7 mutations underlie a neuromuscular junction synaptopathy. Science 2006;313(5795):1975-1978.
89. Chevessier F, Faraut B, Ravel-Chapuis A et al. MUSK, a new target for mutations causing congenital myasthenic syndrome. Hum Mol Genet 2004;13(24):3229-3240.
90. Ohno K, Engel AG, Shen XM et al. Rapsyn mutations in humans cause endplate acetylcholine-receptor deficiency and myasthenic syndrome. Am J Hum Genet 2002;70(4):875-885.
91. Huzé C, Bauché S, Richard P et al. Identification of an agrin mutation that causes congenital myasthenia and affects synapse function. Am J Hum Genet 2009;85(2):155-167.
92. Sanders DB, El Salem K, Massey JM, McConville J, Vincent A. Clinical aspects of MuSK antibody positive seronegative MG. Neurology 2003;60(12):1978-1980.
93. McConville J, Farrugia ME, Beeson D et al. Detection and characterization of MuSK antibodies in seronegative myasthenia gravis. Ann Neurol 2004;55(4):580-584.
94. Romi F, Aarli JA, Gilhus NE. Seronegative myasthenia gravis: disease severity and prognosis. Eur J Neurol 2005;12(6):413-418.
95. Yeh JH, Chen WH, Chiu HC, Vincent A. Low frequency of MuSK antibody in generalized seronegative myasthenia gravis among Chinese. Neurology 2004;62(11):2131-2132.
96. Wirtz PW, Nijnuis MG, Sotodeh M et al. The epidemiology of myasthenia gravis, Lambert-Eaton myasthenic syndrome and their associated tumours in the northern part of the province of South Holland. J Neurol 2003;250(6):698-701.
97. Phillips LH, Torner JC. Epidemiologic evidence for a changing natural history of myasthenia gravis. Neurology 1996;47(5):1233-1238.
98. Tron F, Gilbert D, Mouquet H et al. Genetic factors in pemphigus. J Autoimmun 2005;24(4):319-328.
99. Niks EH, Kuks JBM, Roep BO et al. Strong association of MuSK antibody-positive myasthenia gravis and HLA-DR14-DQ5. Neurology 2006;66(11):1772-1774.
100. Higuchi O, Hamuro J, Motomura M, Yamanashi Y. Autoantibodies to low-density lipoprotein receptor-related protein 4 in myasthenia gravis. Ann Neurol 2011;69(2):418-422.

101. Pevzner A, Schoser B, Peters K et al. Anti-LRP4 autoantibodies in AChR- and MuSK-antibody-negative myasthenia gravis. J Neurol 2011;259(3):427-435.
102. Zhang B, Tzartos JS, Belimezi M et al. Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. Arch Neurol 2011;69(4):445-451.
103. Vincent A, Bowen J, Newsom-Davis J, McConville J. Seronegative generalised myasthenia gravis: clinical features, antibodies, and their targets. Lancet Neurol 2003;2(2):99-106.
104. Lavrnic D, Losen M, Vujic A et al. The features of myasthenia gravis with autoantibodies to MuSK. J Neurol Neurosurg Psychiatry 2005;76(8):1099-1102.
105. Padua L, Tonali P, Aprile I, Caliandro P, Bartoccioni E, Evoli A. Seronegative myasthenia gravis: comparison of neurophysiological picture in MuSK+ and MuSK- patients. Eur J Neurol 2006;13(3):273-276.
106. Deymeer F, Gungor-Tuncer O, Yilmaz V et al. Clinical comparison of anti-MuSK- vs anti-AChR-positive and seronegative myasthenia gravis. Neurology 2007;68(8):609-611.
107. Ohta K, Shigemoto K, Fujinami A, Maruyama N, Konishi T, Ohta M. Clinical and experimental features of MuSK antibody positive MG in Japan. Eur J Neurol 2007;14(9):1029-1034.
108. Huang YC, Yeh JH, Chiu HC, Chen WH. Clinical Characteristics of MuSK Antibody-positive Myasthenia Gravis in Taiwan. J Formos Med Assoc 2008;107(7):572-575.
109. Pasnoor M, Wolfe GI, Nations S et al. Clinical findings in MuSK-antibody positive myasthenia gravis: A U.S. experience. Muscle Nerve 2010;41(3):370-374.
110. Guptill JT, Sanders DB, Evoli A. Anti-MuSK antibody myasthenia gravis: clinical findings and response to treatment in two large cohorts. Muscle Nerve 2011;44(1):36-40.
111. Zhou L, McConville J, Chaudhry V et al. Clinical comparison of muscle-specific tyrosine kinase (MuSK) antibody-positive and -negative myasthenic patients. Muscle Nerve 2004;30(1):55-60.
112. Stickler DE, Massey JM, Sanders DB. MuSK-antibody positive myasthenia gravis: Clinical and electrodiagnostic patterns. Clin Neurophysiol 2005;116(9):2065-2068.
113. Jaretzki A, Barohn RJ, Ernstoff RM et al. Myasthenia gravis: recommendations for clinical research standards. Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America. Neurology 2000;55(1):16-23.
114. Nemoto Y, Kuwabara S, Misawa S et al. Patterns and severity of neuromuscular transmission failure in seronegative myasthenia gravis. J Neurol Neurosurg Psychiatry 2005;76(5):714-718.

115. Niks EH, Kuks JB, Verschuuren JJ. Epidemiology of myasthenia gravis with anti-muscle specific kinase antibodies in The Netherlands. J Neurol Neurosurg Psychiatry 2007;78(4):417-418.
116. Farrugia ME, Robson MD, Clover L et al. MRI and clinical studies of facial and bulbar muscle involvement in MuSK antibody-associated myasthenia gravis. Brain 2006;129(Pt 6):1481-1492.
117. Muller JS, Baumeister SK, Rasic VM et al. Impaired receptor clustering in congenital myasthenic syndrome with novel RAPSN mutations. Neurology 2006;67(7):1159-1164.
118. Alseth EH, Maniaol AH, Elsais A et al. Investigation for RAPSN and DOK-7 mutations in a cohort of seronegative myasthenia gravis patients. Muscle Nerve 2011;43(4):574-577.
119. Verduyn W, Doxiadis II, Anholts J et al. Biotinylated DRB sequence-specific oligonucleotides. Comparison to serologic HLA-DR typing of organ donors in eurotransplant. Hum Immunol 1993;37(1):59-67.
120. Schipper RF, Schreuder GM, D'Amaro J, Oudshoorn M. HLA gene and haplotype frequencies in Dutch blood donors. Tissue Antigens 1996;48(5):562-574.
121. Ahmed AR, Wagner R, Khatri K et al. Major histocompatibility complex haplotypes and class II genes in non-Jewish patients with pemphigus vulgaris. Proc Natl Acad Sci USA 1991;88(11):5056-5060.
122. Delgado JC, Hameed A, Yunis JJ et al. Pemphigus vulgaris autoantibody response is linked to HLA-DQB1*0503 in Pakistani patients. Hum Immunol 1997;57(2):110-119.
123. Bhol K, Natarajan K, Nagarwalla N, Mohimen A, Aoki V, Ahmed AR. Correlation of peptide specificity and IgG subclass with pathogenic and nonpathogenic autoantibodies in pemphigus vulgaris: A model for autoimmunity. Proc Natl Acad Sci USA 1995;92(11):5239-5243.
124. Jha S, Xu K, Maruta T et al. Myasthenia gravis induced in mice by immunization with the recombinant extracellular domain of rat muscle-specific kinase (MuSK). J Neuroimmunol 2006;175(1-2):107-117.
125. Shiraishi H, Motomura M, Yoshimura T et al. Acetylcholine receptors loss and postsynaptic damage in MuSK antibody-positive myasthenia gravis. Ann Neurol 2005;57(2):289-293.
126. Selcen D, Fukuda T, Shen XM, Engel AG. Are MuSK antibodies the primary cause of myasthenic symptoms? Neurology 2004;62(11):1945-1950.
127. Brooke MH. A Clinician's View of Neuromuscular Diseases. 2 ed. Baltimore: Williams & Wilkins; 1986.

128. Bartoccioni E, Scuderi F, Minicuci GM, Marino M, Ciaraffa F, Evoli A. Anti-MuSK antibodies: Correlation with myasthenia gravis severity. Neurology 2006;67(3):505-507.
129. Aalberse RC, van der Gaag R, van Leeuwen J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. J Immunol 1983;130(2):722-726.
130. Matsumoto T, Shima M, Fukuda K et al. Immunological characterization of factor VIII autoantibodies in patients with acquired hemophilia A in the presence or absence of underlying disease. Thromb Res 2001;104(6):381-388.
131. Hillman M, Törn C, Thorgeirsson H, Landin-Olsson M. IgG4-subclass of glutamic acid decarboxylase antibody is more frequent in latent autoimmune diabetes in adults than in type 1 diabetes. Diabetologia 2004;47(11):1984-1989.
132. Hamano H, Kawa S, Horiuchi A et al. High serum IgG4 concentrations in patients with sclerosing pancreatitis. N Engl J Med 2001;344(10):732-738.
133. Futei Y, Amagai M, Ishii K, Kuroda-Kinoshita K, Ohya K, Nishikawa T. Predominant IgG4 subclass in autoantibodies of pemphigus vulgaris and foliaceus. J Dermatol Sci 2001;26(1):55-61.
134. Chan KH, LaChance DH, Harper CM, Lennon VA. Frequency of seronegativity in adult-acquired generalized myasthenia gravis. Muscle Nerve 2007;36(5):651-658.
135. Muppidi S, Wolfe GI. Muscle-specific receptor tyrosine kinase antibody-positive and seronegative myasthenia gravis. Front Neurol Neurosci 2009;26:109-119.
136. Drachman DB. Myasthenia gravis. N Engl J Med 1994;330(25):1797-1810.
137. Jo SA, Zhu X, Marchionni MA, Burden SJ. Neuregulins are concentrated at nerve-muscle synapses and activate ACh-receptor gene expression. Nature 1995;373(6510):158-161.
138. Rimer M. Neuregulins: Primary or secondary signals for the control of synapse-specific gene expression. J Neurocytol 2003;32(5-8):665-675.
139. Zhu X, Lai C, Thomas S, Burden SJ. Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses. EMBO J 1995;14(23):5842-5848.
140. Buonanno A, Fischbach GD. Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr Opin Neurobiol 2001;11(3):287-296.
141. Sanes JR, Lichtman JW. Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 1999;22:389-442.
142. Lemke G. Neuregulins in development. Mol Cell Neurosci 1996;7(4):247-262.
143. Trinidad JC, Fischbach GD, Cohen JB. The Agrin/MuSK signaling pathway is spatially segregated from the neuregulin/ErbB receptor signaling pathway at the neuromuscular junction. J Neurosci 2000;20(23):8762-8770.

144. Sandrock A, Dryer S, Rosen K et al. Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo. Science 1997;276:599-603.
145. Kummer TT, Misgeld T, Sanes JR. Assembly of the postsynaptic membrane at the neuromuscular junction: paradigm lost. Curr Opin Neurobiol 2006;16(1):74-82.
146. Mencarelli C, Hammels C, Van Den Broeck J et al. The expression of the Goodpasture antigen-binding protein (ceramide transporter) in adult rat brain. J Chem Neuroanat 2009;38(2):97-105.
147. Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN. The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. Oncologist 2009;14(4):320-368.
148. Cobleigh MA, Vogel CL, Tripathy D et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 1999;17(9):2639-2648.
149. Baselga J, Tripathy D, Mendelsohn J et al. Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. Semin Oncol 1999;26(4 Suppl 12):78-83.
150. Le PR, Cizeron-Clairac G, Bismuth J, Berrih-Aknin S. Microarrays reveal distinct gene signatures in the thymus of seropositive and seronegative myasthenia gravis patients and the role of CC chemokine ligand 21 in thymic hyperplasia. J Immunol 2006;177(11):7868-7879.
151. Cizeron-Clairac G, Le PR, Frenkian-Cuvelier M et al. Thymus and Myasthenia Gravis: what can we learn from DNA microarrays? J Neuroimmunol 2008;201-202:57-63.
152. Lefvert AK, Osterman PO. Newborn infants to myasthenic mothers: a clinical study and an investigation of acetylcholine receptor antibodies in 17 children. Neurology 1983;33(2):133-138.
153. Ahlsten G, Lefvert AK, Osterman PO, Stålberg E, Säfwenberg J. Follow-up study of muscle function in children of mothers with myasthenia gravis during pregnancy. J Child Neurol 1992;7(3):264-269.
154. Morel E, Eymard B, Vernet-der Garabedian B, Pannier C, Dulac O, Bach JF. Neonatal myasthenia gravis: a new clinical and immunologic appraisal on 30 cases. Neurology 1988;38(1):138-142.
155. Tzartos SJ, Efthimiadis A, Morel E, Eymard B, Bach JF. Neonatal myasthenia gravis: antigenic specificities of antibodies in sera from mothers and their infants. Clin Exp Immunol 1990;80(3):376-380.
156. Malek A, Sager R, Kuhn P, Nicolaides KH, Schneider H. Evolution of maternofetal transport of immunoglobulins during human pregnancy. Am J Reprod Immunol 1996;36(5):248-255.

157. Vincent A, Leite MI, Farrugia ME et al. Myasthenia gravis seronegative for acetylcholine receptor antibodies. Ann N Y Acad Sci 2008;1132:84-92.
158. Farrugia ME, Bonifati DM, Clover L, Cossins J, Beeson D, Vincent A. Effect of sera from AChR-antibody negative myasthenia gravis patients on AChR and MuSK in cell cultures. J Neuroimmunol 2007;185(1-2):136-144.
159. Song Y, Balice-Gordon R. New dogs in the dogma: Lrp4 and Tid1 in neuromuscular synapse formation. Neuron 2008;60(4):526-528.
160. Strochlic L, Cartaud A, Cartaud J. The synaptic muscle-specific kinase (MuSK) complex: new partners, new functions. Bioessays 2005;27(11):1129-1135.
161. Niks EH, van Leeuwen Y, Leite MI et al. Clinical fluctuations in MuSK myasthenia gravis are related to antigen-specific IgG4 instead of IgG1. J Neuroimmunol 2008;195(1-2):151-156.
162. Plomp JJ, Van Kempen GT, De Baets MB, Graus YM, Kuks JB, Molenaar PC. Acetylcholine release in myasthenia gravis: regulation at single end-plate level. Ann Neurol 1995;37(5):627-636.
163. Pestronk A, Drachman DB. A new stain for quantitative measurement of sprouting at neuromuscular junctions. Muscle Nerve 1978;1(1):70-74.
164. Elmqvist D, Hofmann WW, Kugelberg J, Quastel DM. An electrophysiological investigation of neuromuscular transmission in myasthenia gravis. J Physiol 1964;174:417-434.
165. Cull-Candy SG, Miledi R, Trautmann A, Uchitel OD. On the release of transmitter at normal, myasthenia gravis and myasthenic syndrome affected human end-plates. J Physiol 1980;299:621-638.
166. Shigemoto K, Kubo S, Maruyama N et al. Induction of myasthenia by immunization against muscle-specific kinase. J Clin Invest 2006;116(4):1016-1024.
167. Slater CR, Lyons PR, Walls TJ, Fawcett PR, Young C. Structure and function of neuromuscular junctions in the vastus lateralis of man. A motor point biopsy study of two groups of patients. Brain 1992;115:451-478.
168. Ip FC, Glass DG, Gies DR et al. Cloning and characterization of muscle-specific kinase in chicken. Mol Cell Neurosci 2000;16(5):661-673.
169. Maselli RA, Wollman RL, Leung C et al. Neuromuscular transmission in amyotrophic lateral sclerosis. Muscle Nerve 1993;16(11):1193-1203.
170. Tsujihata M, Hazama R, Yoshimura T, Satoh A, Mori M, Nagataki S. The motor end-plate fine structure and ultrastructural localization of acetylcholine receptors in amyotrophic lateral sclerosis. Muscle Nerve 1984;7(3):243-249.
171. Cole RN, Reddel SW, Gervásio OL, Phillips WD. Anti-MuSK patient antibodies disrupt the mouse neuromuscular junction. Ann Neurol 2008;63(6):782-789.

172. Chang T, Gunaratne P, Gamage R, Riffsy MTM, Vincent A. MuSK-antibody-positive myasthenia gravis in a South Asian population. J Neurol Sci 2009;284(1-2):33-35.
173. Oh SJ, Morgan MB, Lu L et al. Racial differences in myasthenia gravis in Alabama. Muscle Nerve 2009;39(3):328-332.
174. Behin A, Mayer M, Kassis-Makhoul B et al. Severe neonatal myasthenia due to maternal anti-MuSK antibodies. Neuromuscular Disorders 2008;18(6):443-446.
175. Murray EL, Kedar S, Vedanarayanan VV. Transmission of maternal muscle-specific tyrosine kinase (MuSK) to offspring: report of two cases. J Clin Neuromuscul Dis 2010;12(2):76-79.
176. O'Carroll P, Bertorini TE, Jacob G, Mitchell CW, Graff J. Transient neonatal myasthenia gravis in a baby born to a mother with new-onset anti-MuSK-mediated myasthenia gravis. J Clin Neuromuscul Dis 2009;11(2):69-71.
177. Mori S, Yamada S, Kubo S et al. Divalent and monovalent autoantibodies cause dysfunction of MuSK by distinct mechanisms in a rabbit model of myasthenia gravis. J Neuroimmunol 2012.
178. Klooster R, Plomp JJ, Huijbers MG et al. Muscle-specific kinase myasthenia gravis IgG4 autoantibodies cause severe neuromuscular junction dysfunction in mice. Brain 2012;135(4):1081-1101.
179. Kaneko Y, Nimmerjahn F, Ravetch JV. Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science 2006;313(5787):670-673.
180. Van de Geijn FE, Wuhrer M, Selman MH et al. Immunoglobulin G galactosylation and sialylation are associated with pregnancy-induced improvement of rheumatoid arthritis and the postpartum flare: results from a large prospective cohort study. Arthritis Res Ther 2009;11(6):R193.
181. Selman MH, Niks EH, Titulaer MJ, Verschuuren JJ, Wuhrer M, Deelder AM. IgG fc N-glycosylation changes in Lambert-Eaton myasthenic syndrome and myasthenia gravis. J Proteome Res 2011;10(1):143-152.
182. Diaz-Manera J, Martinez-Hernandez E, Querol L et al. Long-lasting treatment effect of rituximab in MuSK myasthenia. Neurology 2012;78(3):189-193.
183. Perosa F, Prete M, Racanelli V, Dammacco F. CD20-depleting therapy in autoimmune diseases: from basic research to the clinic. J Intern Med 2010;267(3):260-277.
184. Liewluck T, Selcen D, Engel AG. Beneficial effects of albuterol in congenital endplate acetylcholinesterase deficiency and Dok-7 myasthenia. Muscle Nerve 2011;44(5):789-794.

185. Lashley D, Palace J, Jayawant S, Robb S, Beeson D. Ephedrine treatment in congenital myasthenic syndrome due to mutations in DOK7. Neurology 2010;74(19):1517-1523.
186. Ryall JG, Church JE, Lynch GS. Novel role for β-adrenergic signalling in skeletal muscle growth, development and regeneration. Clin Exp Pharmacol Physiol 2010;37(3):397-401.
187. Maselli RA, Ng JJ, Anderson JA et al. Mutations in LAMB2 causing a severe form of synaptic congenital myasthenic syndrome. J Med Genet 2009;46(3):203-208.
188. Provenzano C, Marino M, Scuderi F, Evoli A, Bartoccioni E. Anti-acetylcholinesterase antibodies associate with ocular myasthenia gravis. J Neuroimmunol 2010;218(1-2):102-106.
189. Romi F, Suzuki S, Suzuki N, Petzold A, Plant GT, Gilhus NE. Anti-voltage-gated potassium channel Kv1.4 antibodies in myasthenia gravis. J Neurol 2011;259(7):1312-1316.
190. Kaminski HJ, Richmonds CR, Kusner LL, Mitsumoto H. Differential susceptibility of the ocular motor system to disease. Ann N Y Acad Sci 2002;956:42-54.
191. Punga AR, Maj M, Lin S, Meinen S, Ruegg MA. MuSK levels differ between adult skeletal muscles and influence postsynaptic plasticity. Eur J Neurosci 2011;33(5):890-898.