

**CLINICAL AND FUNCTIONAL
CHARACTERISTICS OF SERONEGATIVE
AND MUSK MYASTHENIA GRAVIS**



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Abstract

Myasthenia gravis (MG) is an autoimmune disorder of the neuromuscular junction (NMJ). Most patients have antibodies (Abs) against the acetylcholine receptor (AChR) or muscle specific kinase (MuSK) detected by radioimmunoassays (RIAs). Cell-based assays can more sensitively detect these and other Abs (e.g. to low density lipoprotein receptor-related protein 4 (LRP4)) but no large cohorts of MG patients have been systematically studied. RIA positive MuSK-Abs are mainly IgG4 subclass and block the interaction between LRP4 and MuSK, resulting in reduced MuSK phosphorylation and agrin-induced AChR clustering at the NMJ. MuSK phosphorylation could provide a potential therapeutic target.

An international cohort of 231 MG patients negative for AChR and MuSK Abs by RIA, were tested with CBAs for all three known antigens. Negative sera were screened for novel antigenic targets by immunoprecipitation and mass spectrometry (IP/MS). MuSK phosphorylation was assessed in cultured mouse C2C12 myotubes treated with MuSK-MG IgG subclass fractions. The formation, dispersal, and re-formation of AChR clusters by MuSK-Abs in the presence or absence of a tyrosine phosphatase SHP2 inhibitor (NSC-87877) were measured quantitatively.

First, the detection of MuSK-IgG Abs by CBA was optimised, and the potential relevance of the antibodies shown by their ability to reduce agrin-induced AChR clusters in the myotubes. Overall, AChR-Abs (10%), MuSK-Abs (7%) and LRP4-Abs (<1%) antibodies were newly identified. Five negative sera bound to human muscle cell lines, but no plausible novel antigens were identified by IP/MS. MuSK-MG IgG4 fractions inhibited MuSK phosphorylation, which was restored by SHP2 inhibition. SHP2 inhibition also increased AChR cluster formation, blocked dispersal and increased re-formation of AChR clusters by MuSK-Abs.

As discussed, finding new antibody targets in MG was confounded by testing patients who were no longer severely affected following immunotherapies. MuSK-MG, by contrast, is frequently severe and modifying phosphorylation of MuSK and its downstream signalling pathways may provide a novel treatment approach for this and other forms of MG.

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Abbreviations and Acronyms

A	azathioprine	CPM	counts per minute
ABL	Abelson murine leukemia viral oncogene homolog 1	CRD	cystein-rich domain
Abs	antibody	Crk	CT10 regulator of kinase
AChEI	cholinesterase inhibitor	CSR	complete stable remission
AChR	acetylcholine receptor	cyclph	cyclophosphamide
AChR	acetylcholine receptor	cyclosp	cyclosporin
AKT	protein kinase B	Cys	carbamidomethylation
ALMD	agrin/LRP4/MuSK/DOK7 pathway	DAPI	4'6-diamidino-2-phenylindole
APC	antigen presenting cell	DEP	dishevelled/Egl-10/pleckstrin
APP	amyloid precursor protein	DMEM	Dulbecco's modified eagle's medium
APS	antiphospholipid syndrome	DOK7	docking protein 7
AQP4	aquaporin-4	Dvl1	dishevelled-1
β -pd	β -propellor domain	EAMG	experimental autoimmune myasthenia gravis
BSA	bovine serum albumin	EDTA	ethylenediaminetetraacetic acid
BTX	bungaratoxin	EGF	epidermal growth factor
cAChR	clustered acetylcholine receptor	ELISA	enzyme linked immunosorbent assay
CamKII	Calcium/calmoduline-dependent protein kinase II	EOMG	early onset myasthenia gravis
CBA	cell-based assay	EPP	end-plate potential
CBA	cell-based assay	ERK	extracellular signal-regulated kinase
CDK5	cyclin-dependent kinase 5	ESI	electrospray ionisation
CID	collision induced dissociation	F	female
CK2	casein kinase 2	Fab	fragment antigen binding
CLASP2	cytoplasmic linker associated protein 2	FACS	flourescent activated cell sorting
CLIP2	CAP-gly domain containing linker protein 2	FAK	focal adhesion kinase
CMAP	compound muscle action potential	Fc	fragment crystallisable
Col-q	collagen-q	FCS	foetal calf serum
COPD22	Combined oxidative phosphorylation deficiency 22	Fc γ R	Fc gamma receptor

FS	follistatin domain	MASC	myotube associated specificity component
Ger	Germany	MEPP	miniature end plate potential
GFP	green fluorescent protein	Met	methylation
GGT	geranylgeranyl transferase	MG	myasthenia gravis
GMG	generalised myasthenia gravis	MGFA	Myasthenia Gravis Foundation America
GSK-3	glycogen synthase kinase 3	MHC	major histocompatibility complex
H	heavy	min	minute
H+L	heavy and light chains	MM	minimal manifestations
HC	healthy control	MOG	myelin oligodendrocyte glycoprotein
HEK	human embryonic kidney	MR	medial rectus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	MS	mass spectrometry
HFD	Hyperthyroxinaemia familial dysalbuminaemic	MS/MS	tandem mass spectrometry
HLA	human leukocyte antigen	MuSK	muscle specific kinase
HPLC	high performance liquid chromatography	NCS/EMG	nerve conduction studies/electromyography
HRP	horseradish peroxidase	NMDAR	N-methyl-D-aspartate receptor
hrs	hours	NMJ	neuromuscular junction
I	improved	non-MuSK-MG	indistinguishable from non-MuSK-MG
Ig	immunoglobulin	Nor	Norway
Ig	immunoglobulin	NtA	amino terminal domain
IP	immunoprecipitation	OMG	ocular myasthenia gravis
Ita	Italy	P	prednisone
JAK-STAT	Janus kinase/signal transducers and activators of transcription	PAK1	p21-activated kinase
JHN	Familial juvenile hyperuricaemic nephropathy 1	PBS	phosphate buffered saline
JMD	juxtamembrane domain	PDZRN3	PDZ domain containing ring finger 3
kDa	kilodalton	PEI	polyethylenimine
L	light	PH	pleckstrin homology
LB	laminin- β -like modules	Phi	Philippines
LDLa	low density lipoprotein receptor domain class A	PI3K	phosphoinositide 3-kinase
LDLR	low density lipoprotein receptor	PIS	post-intervention status
LDS	lithium dodecyl sulfate	PIS	post-intervention status
LG	laminin globulin	PKA	protein kinase A
LL5 β	pleckstrin homology-like domain family B member	PKC	protein kinase C
LOMG	late onset myasthenia gravis	PR	pharmacological remission
LRP4	low-density lipoprotein receptor-related protein 4	PTB	phosphotyrosine binding domain
M	male	PTP	protein tyrosine phosphatase
MALDI	matrix-assisted laser desorption/ionisation	PTX	phosphate buffer saline containing triton-X

RA	rheumatoid arthritis	SMN	survival motor neuron
resp	respiratory	SNMG	seronegative myasthenia gravis
RIA	radioimmunoassay	TID1	tumourous imaginal disc 1
RIPA	radioimmunoprecipitation assay buffer	TKD	tyrosine kinase domain
Ritux	rituximab	TMD	transmembrane domain
RNS	repetitive nerve stimulation	TRIM21	tripartite motif-containing protein 21
RT	room temperature	Tur	Turkey
S/T	serine-threonine	Tyr	tyrosine
SD	standard deviation	U	unresponsive
SDS- PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis	UK	United Kingdom
SEM	standard error of the mean	VDJ	variable/diverse/joining
SHP2	src homology 2 domain containing phosphotyrosine phosphatase	WNT	wingless
SLE	systemic lupus erythematosus	Unk	unknown
SK	South Korea	Utr	Utrophin

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Chapter 1: Introduction

1.1 Autoimmune disease

Up until the 20th century, the idea that the immune system was able to react against the body's own tissues was an unfamiliar concept. In fact this notion did not gain widespread acceptance until the 1960's. In 1900, Ehrlich's "horror autotoxicus" challenged the autoimmune concept on the basis that goats immunised with their own red blood cells did not develop autoimmune disease, and did so only weakly when immunised with blood cells from animals of the same species (Silverstein 2001). These observations reflected the important concept of immune tolerance but at the time were interpreted as evidence against autoimmune disease. Interestingly Bordet two years previously had observed haemolytic effects in goat sera following immunisation with red blood cells of other species (Mackay 2010). Further evidence for autoimmune disease was provided in Donath and Landsteiner's report of autohaemolysin in the blood of three patients with cold-induced haemoglobinuria (Donath & Landsteiner 1925). Subsequent studies involving experimental induction of thyroiditis using thyroglobulin, and the identification of thyroglobulin antibodies in patients with Hashimoto's thyroiditis (Roitt et al. 1956; Witebsky et al. 1957; Rose & Witebsky 1956), helped to substantiate autoimmunity as a cause of human disease (reviewed by Mackay 2010). An in-depth review of the immune system is beyond the scope of this thesis but a basic introduction of concepts that are relevant to MG follows.

1.2 Immune system

The innate and adaptive responses of the immune system are designed to protect the body from invading pathogens. Regulation of this function is critically dependent upon mechanisms of immune tolerance (e.g. clonal deletion of auto-reactive B- and T-cells) and simplistically, the ability to distinguish between 'self' and 'non-self' molecules. In myasthenia gravis, as with other autoimmune diseases this regulation is impaired resulting in an adaptive immune response against 'self'-antigens.

The adaptive arm of the immune system is mediated by B- and T-lymphocytes, and during development several immune tolerance checkpoints exist for deletion (negative selection) of immature cells that display auto-recognition. Activation of mature lymphocytes typically occurs through an antigen-presenting cell (APC) that displays antigen fragments associated with major histocompatibility complex (MHC) I or II. MHC I is present on all nucleated cells to present internal peptides and MHC II is normally found on APCs such as dendritic cells. The diverse lymphocyte receptor (e.g. B-cell and T-cell receptors) repertoire allows for recognition of virtually any antigen, and is largely facilitated by variable/diversity/joining region recombination and somatic hypermutation. By contrast the innate immune system relies upon more primitive pattern recognition receptors on (for example) macrophages for the detection of pathogens.

B-cells bind target antigens through B-cell receptors. The antigen is engulfed and then expressed on the cell surface in combination with MHC II. This induces recognition by T-helper cells which provide co-stimulation to maximally activate the B-cell. Upon activation the B-cell enlarges, divides, and differentiates into a clone of plasma cells. Importantly the antibodies produced by these clonally selected progeny share the same antigen specificity as the B-cell from which they were derived, ensuring a targeted response. In addition memory B-cells are produced, which are important for secondary immune responses. In particular CD27⁺ memory B-cells have been suggested to be a useful marker

of disease activity following B-cell targeted therapy with Rituximab in certain forms of MG (Lebrun et al. 2016).

1.3 Antibody isotype

In humans there are five antibody or immunoglobulin isotypes, IgA, IgD, IgE, IgG, and IgM which differ in localisation, structure and effector function. IgM antibodies (Abs) are the first immunoglobulin type produced by B-cells in a humoral response (Janeway et al. 2001). IgM Abs are of low affinity as they are produced prior to somatic hypermutation, but exhibit high overall avidity due to a pentameric structure with 10 antigen-binding sites. IgM is also a potent activator of the complement system, which is described below. IgA has two isotypes: IgA1 is primarily found in serum, whilst secretory IgA2 can be found in respiratory and intestinal secretions. IgD is co-expressed with IgM on mature B-cells and is presumed to have pro-inflammatory functions, though its role is not fully understood (Chen & Cerutti 2011). IgE is mostly bound to mast cells, and has an essential role in type 1 hypersensitivity reactions. Most neurological autoimmune diseases are caused by IgG, which is the most abundant immunoglobulin in human serum and accounts for 10-20% of plasma protein. IgG consists of four subclasses listed in order of decreasing concentration; IgG1, IgG2, IgG3, and IgG4.

1.4 IgG structure and effector function

All immunoglobulins including IgG have a similar Y-shaped structure consisting of two identical heavy (50kDa) polypeptide chains (α , δ , ϵ , γ , μ) and two identical light (25kDa) polypeptide chains (κ , λ ; **Figure 1.1**). The heavy (H) and light (L) chains are linked together by disulphide bonds as are the two H-chains. The number of inter-chain disulphide bonds varies between different IgG subclasses. The (Fab) region is formed by one constant and one variable domain of each of the H- and L- chains. Antigen-binding specificity is

determined by the variable domains of one H-chain and L-chain. As mentioned above, much of the diversity of antigen specificity is made possible through differential assembly of V, D, and J regions as well as somatic mutations. The Fragment crystalline (Fc) region is formed by the second and third constant domains of the H-chain (CH2/CH3). The CH2 domain contains overlapping binding sites for C1q (complement) and IgG-Fc receptors (FcγRs). There are multiple FcγRs which are involved in antibody mediated cellular cytotoxicity, and the interaction varies between different IgG subclasses (Vidarsson et al. 2014).

There is over 90% homology in amino acid sequences between IgG subclasses with the majority of differences found in the hinge regions and N-terminal CH2 domains. These account for the functional differences in (for instance) opsonisation between the subclasses and are summarised in **Table 1.1**. IgG1 and IgG3 efficiently activate the complement pathway, whereas IgG2 does so less efficiently, and IgG4 binds to C1q poorly. The explanation for the latter finding has been attributed to the substitution of serine-331 for proline (P331S) in the CH2 domain of IgG4 (Brekke et al. 1994). Importantly, another serine residue (S228) in the IgG4 hinge region allows for the formation of ‘half-molecules’ facilitating Fab-arm exchange *in vivo*, which results in monovalent antibodies of dual specificity (van der Zee et al. 1986; van der Neut Kolfshoten et al. 2007; Petersen & Dorrington 1974; Angal et al. 1993; Schuurman et al. 2001).

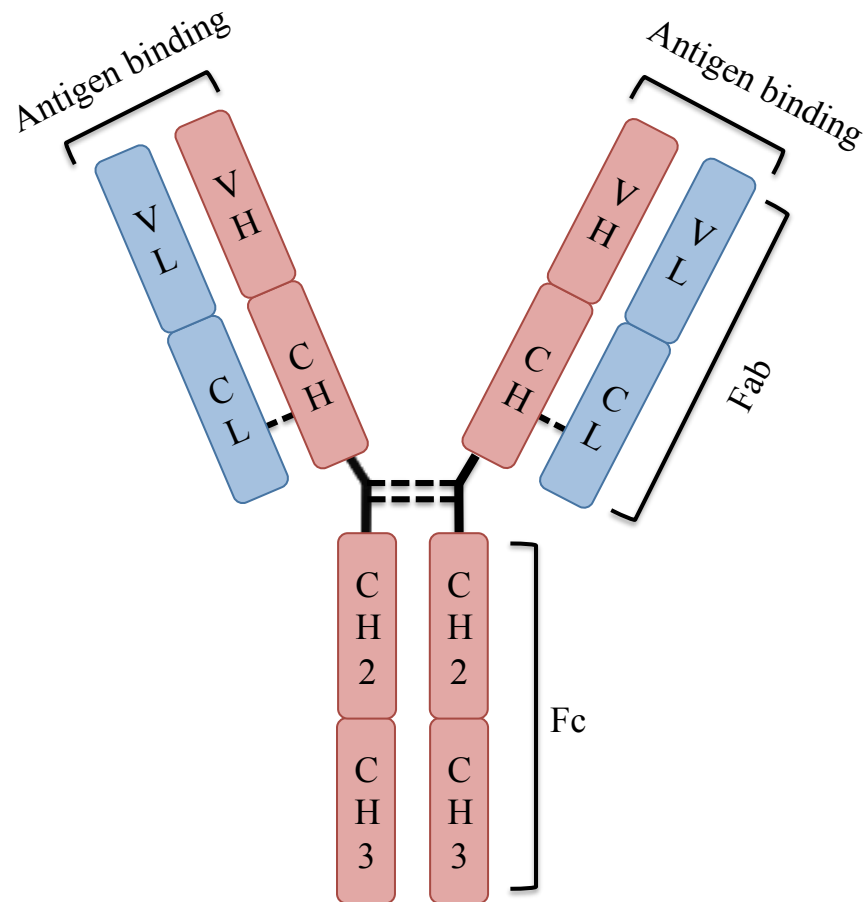


Figure 1.1. Structure of IgG

Subclass	Mass (kDa)	Serum range mg/ml	% of total IgG	Opsonisation	C1q binding
IgG1	146	5-11	43-75	+++	++
IgG2	146	1.5-6	16-48	+	+/-
IgG3	170	0.2-1	1.7-7	++	+++
IgG4	146	0.08-1.4	0.8-11.7	+	-

Table 1.1. Characteristics of human IgG subclasses- (adapted from Nirula et al. 2011)

1.5 Autoimmune postulates

Ernst Witebsky's postulates have traditionally been used as the criteria for defining an autoimmune disease and have been updated as follows: (1.) Direct evidence of an autoimmune response mediated by pathogenic antibodies or T-cells, (2.) Indirect evidence based on experimental induction of autoimmune disease in an animal model, (3.) Circumstantial evidence from clinical clues (Witebsky et al. 1957; Rose & Bona 1993).

Myasthenia gravis (MG) is considered to be a prototypical autoimmune disease. This disorder affects neuromuscular transmission resulting in muscle weakness. Communication between a nerve and muscle occurs across a synapse separating the pre-synaptic nerve terminal from the post-synaptic muscle end-plate (**Figure 1.2**). Neuromuscular transmission occurs through the neurotransmitter acetylcholine (ACh), which is released from pre-synaptic vesicles, crosses the synaptic cleft, and binds to acetylcholine receptors (AChR) at the muscle end plate. The opening of AChR ion channels leads to depolarisation and an end plate potential (EPP). Normally the EPP exceeds the threshold potential required for activation of voltage gated sodium channels, resulting in propagation of an action potential. The margin by which the EPP exceeds the threshold potential is termed the 'safety factor'. In addition synaptic vesicles spontaneously release ACh molecules, which result in miniature end-plate potentials (MEPP), and reflect the post-synaptic AChR density (Katz & Miledi 1965; Fatt & Katz 1952). The structure, function, and electrophysiological characteristics of the neuromuscular junction will be described in more detail below.

In MG, pathogenic AChR antibodies (Abs) are found in the majority of patients (Lindstrom et al. 1976; Vincent & Newsom-Davis 1985), the disease can be transferred to animals (Toyka et al. 1975), and patients improve with plasma exchange and immunotherapies (Newsom-Davis et al. 1978), thus fulfilling Witebsky's postulates. More recently Abs targeting surface expressed (and therefore accessible) proteins critical to neuromuscular transmission have also been identified, including muscle specific kinase

(MuSK; Hoch et al. 2001) and low-density lipoprotein receptor-related protein 4 (LRP4; Pevzner et al. 2012; Zhang et al. 2012; Higuchi et al. 2011).

1.6 Clinical features of myasthenia gravis

MG is a rare disease with a pooled worldwide prevalence rate of 77.7 cases per million of the population but there is apparent heterogeneity depending on geographical location (Carr et al. 2010). Prevalence has increased over time, likely due to improved recognition, diagnosis, and treatment. In addition, life expectancy of both MG patients and the general population has also increased. Ptosis and diplopia are the most common initial presenting symptoms and are present in 85% of patients. In 20% of patients the disease remains confined to the ocular muscles (Grob et al. 2008). The remaining 80% develop facial, bulbar, respiratory, axial or limb muscle involvement signifying generalised disease. Early ocular involvement is thought to be due to a relatively reduced safety factor, complement regulation and simplified postsynaptic structure, and also reflects increased susceptibility to neurotoxins (that block AChR function) in these muscles (Serra et al. 2011; Serra et al. 2012; Sanmuganathan 1998). In addition extraocular muscles are ‘high throughput’ muscles and even mild weakness results in diplopia that is invariably symptomatic. Weakness typically increases with activity and as the day progresses, therefore a diurnal pattern may be noted. Rest and cooler temperatures improve symptoms and the latter can be used as a diagnostic aid in the form of an ‘ice pack test’ for ocular MG.

Table 1.2 summarises the common clinical features of MG. The severity and distribution of clinical weakness is routinely assessed using the MG Foundation of America (MGFA) classification (**Table 1.3**; Jaretzki et al. 2000).

Currently there are no internationally agreed standardised diagnostic criteria for MG. The diagnosis is predominantly made on clinical grounds, and with a characteristic presentation there is little diagnostic uncertainty. However, in some MG subtypes such as

ocular MG, symptoms can remain relatively indolent for long periods of time resulting in a delay to diagnosis. Furthermore, disease mimics may cause diagnostic difficulty in more clinically ambiguous cases (**Table 1.4** and **Table 1.5**). In such cases, additional findings such as decrement on repetitive nerve stimulation (RNS, **Figure 1.3**; upper), jitter/block on single fibre electromyography (**Figure 1.3**; lower), a positive tensilon test, the serological detection of AChR-, MuSK-, or LRP4-Abs, and a beneficial response to immunotherapy/plasma exchange can be diagnostically helpful.

1.7 Classification of myasthenia gravis

MG can be classified according to distribution of affected muscles, age of onset, thymic abnormalities, or antibody status. Within these groups there are variations in gender, phenotype, clinical course and prognosis.

Based on muscle distribution, patients with MG can be divided into those with ocular confined (OMG) or generalised MG (GMG). OMG is usually diagnosed after a minimum duration of 2 years, as 80% of patients will develop GMG within this time frame (Bever et al. 1983; Oosterhuis 1989; Grob et al. 2008). Factors such as AChR-Ab seropositivity, presence of one or more co-morbidities, and thymic hyperplasia increase the risk of generalisation (Wong et al. 2016). Despite there being a female preponderance in MG overall, OMG is more common in men with advancing age (Oosterhuis 1989; Sommer et al. 1993; Wong et al. 2014). The clinical course of OMG is invariably milder than GMG but diplopia can be functionally limiting, particularly with tasks such as driving. Patients with GMG can develop prominent bulbar symptoms or respiratory failure, both of which are medical emergencies. High doses of corticosteroids are often required with the additional use of ‘steroid sparing agents’ such as azathioprine, methotrexate, or mycophenolate mofetil. Rescue treatments such as intravenous immunoglobulins and plasma exchange may also be required, all of which are associated with potentially serious side effects. By contrast OMG may only

require cholinesterase inhibitors (AChEIs) or the addition of a modest dose of corticosteroids to suppress symptoms.

Table 1.6 lists the drugs that are commonly used in the treatment of myasthenia gravis, their mode of action and side effects. The response to treatments in MG is commonly assessed using the postintervention status scale (PIS; **Table 1.7**).

MG peaks between the ages of 25-35 years and 60-80 years rationalising a division of MG into early or late onset MG (EOMG and LOMG; Somnier et al. 1991; Carr et al. 2010; Compston et al. 1980). EOMG refers to patients presenting before the age of 40 years (Compston et al. 1980). These patients are often females, seropositive for AChR-Abs, and have thymic hyperplasia with active germinal centres. There is an association with HLA DR3-B8 in Caucasians and HLA-DR9 in Asian populations (Vandiedonck et al. 2004; Matsuki et al. 1990). The definition of where the division between EOMG and LOMG should be is contentious but ages between 40-65 years have been proposed (Meriggioli & Sanders 2009; Alkhawajah & Oger 2013). Males are more frequently affected, and the disease course can be more severe. There is also a greater risk of complication from treatment and comorbidities are frequently present (Aarli 1999; Donadson et al. 1990). Conversely there is also a subset of late onset male patients with ocular confined disease. In contrast to EOMG, a normal or atrophic thymus is more commonly detected but the risk of thymoma is higher (Aarli 1999). The genetic associations also differ, with LOMG associated with HLA-B7, HLA-DR15 and TNFRSF11A (Maniaol et al. 2012; Seldin & Alkhairy 2015).

Approximately 10-15% of MG patients have a thymoma, a thymic epithelial tumour. Of patients with thymoma as many as 30-50% develop myasthenia gravis (Sommer et al. 1993). The onset of thymoma-associated MG peaks at the age of 50 years but can occur at any age. There are no differences in the incidence rates between men and women. The clinical course is often progressive with bulbar involvement, and severity of disease is greater than EOMG without thymoma. High titres of AChR-Abs are frequently detected as well as antibodies against titin (Skeie & Romi 2008; Compston et al. 1980). In non-thymomatous

MG, a randomised controlled trial supports the use of thymectomy to improve the clinical course of MG.

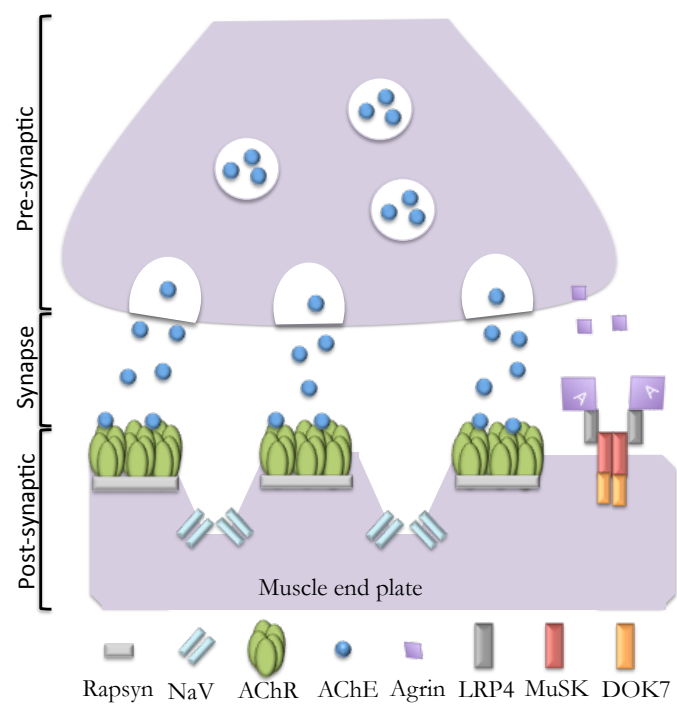


Figure 1.2. The neuromuscular junction. NaV = voltage-gated sodium channel; AChR = acetylcholine receptor; AChE = acetylcholinesterase; LRP4 = low density lipoprotein receptor-related protein 4; MuSK = muscle specific kinase; DOK7 = docking protein-7

Clinical features of myasthenia gravis	
Ocular-	ptosis, diplopia (often MR involved)
Bulbar-	dysphagia, dysarthria, dysphonia, jaw fatigue
Facial-	eye lid closure, ‘myasthenic fascies’
Limb-	proximal symmetrical limb fatigue
Axial-	neck flexors, dropped head
Respiratory-	dyspnoea on exertion, orthopnoea, nocturnal hypoventilation (daytime somnolence/morning headaches)

Table 1.2. Clinical features of myasthenia gravis. MR = medial rectus muscle.

Class	Clinical findings
1	Ocular symptoms only
2	Mild generalised
3	Moderate generalised
4	Severe generalised*
5	Life-threatening-intubation+/-mechanical ventilation
<i>Subdivisions</i>	
A	Predominantly limb and/or axial
B	Predominantly bulbar and/or respiratory

Table 1.3. Myasthenia Foundation of America (MGFA) classification- adapted from (Jaretzki et al. 2000). *; nasogastric tube insertion without intubation.

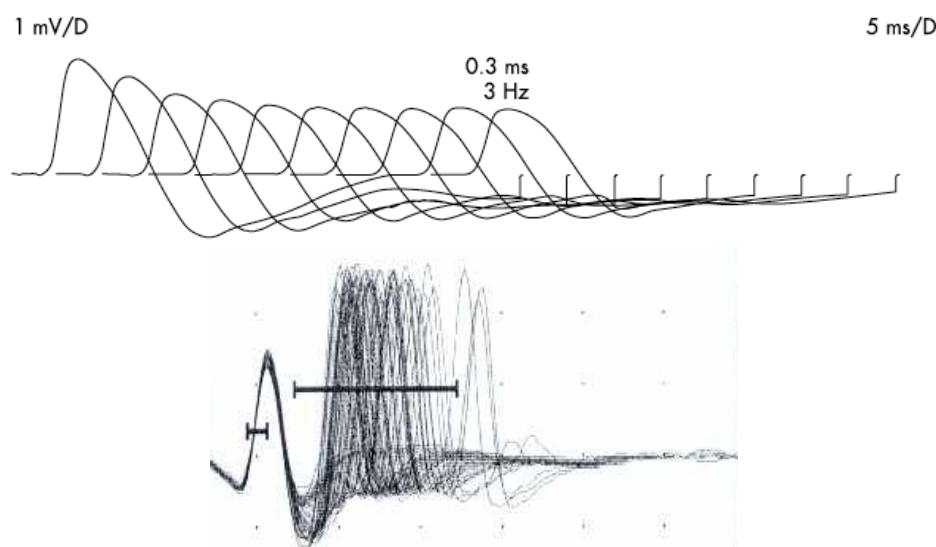


Figure 1.3. Neurophysiological tests for the diagnosis of myasthenia gravis- *Upper:* Repetitive nerve stimulation involves the electrical stimulation (typically 3Hz) of a nerve and its corresponding muscle. Normally the amplitude of summative action potentials from the recorded muscle fibres (Compound Muscle Action Potential; CMAP) is more than sufficient, but when compromised as in the case of MG, decrement (significant decrement is typically defined as >10%) after the 4th or 5th CMAP can be seen. In myasthenia gravis this suggests there is progressive drop of end plate potential (EPP) in measured muscle fibres below the threshold for activation, which in normal muscles is protected by the safety factor.

Lower: Single fibre EMG compares the motor unit action potentials of two muscle fibres subserved by the same motor neuron. In healthy subjects there is minimal delay or interpotential interval between the two muscle fibres. In myasthenia gravis, due to impaired neuromuscular transmission the interpotential interval is increased, and when systematically assessed in 20 fibre pairs the mean of these consecutive differences or jitter value may be elevated. In some cases a second action potential may be unrecordable, reflecting 'blocking' in the affected muscles, analogous to a failure to stimulate beyond the threshold EPP. The electrophysiological properties of the neuromuscular junction are discussed in more detail in **Section 1.19- Neuromuscular Junction**.

Differential diagnosis of ocular MG
Thyroid eye disease
Congenital and acquired strabismus
Chronic progressive external ophthalmoplegia
Single and multiple cranial neuropathies (III, IV, VI)
Levator dehiscence
Orbital inflammatory syndromes
Tolosa-Hunt syndrome
Myotonic dystrophy Type 1

Table 1.4. Disease mimics of ocular myasthenia gravis.

Differential diagnosis of generalised MG
Motor neuron syndromes
Lambert-Eaton syndrome
Myopathy/muscular dystrophies
Mitochondrial cytopathies
Metabolic e.g. thyroid disease, hypomagnesaemia
Botulism
Brainstem pathology e.g. inflammation, ischaemia
Motor neuron syndromes

Table 1.5. Disease mimics of generalised myasthenia gravis.

Treatment	Mechanism of action	Serious side effects
Pyridostigmine	Acetylcholinesterase inhibition	Cholinergic crisis
Prednisone	Suppression of T-cell and humoral mediated immunity	Cushing's syndrome, hypertension, diabetes, osteoporosis, psychiatric, adrenal suppression, gastric ulceration
<i>Steroid 'sparing' agents</i>		
Azathioprine	Inhibits DNA synthesis and cell proliferation	Bone marrow suppression, hepatotoxicity, cancer, pancreatitis
Methotrexate	Inhibits purine and pyrimidine synthesis	Bone marrow suppression, hepatotoxicity, pulmonary fibrosis
Mycophenolate mofetil	Inhibits inosine monophosphate dehydrogenase	Bone marrow suppression, hepatotoxicity, PML
Rituximab	Monoclonal antibody against CD20 of B-cells	Fatal infusion related, neutropaenia, PML
Tacrolimus	Inhibits calcineurin	Diabetes, hypertension, nephrotoxicity, cancer
Ciclosporin	Inhibits calcineurin	Hypertension, nephrotoxicity, cancer
<i>Rescue therapies</i>		
Intravenous immunoglobulin	Unclear- Fc binding to pathogenic Abs, anti-idiotype Abs	Aseptic meningitis, renal failure, volume overload, thrombosis
Plasma exchange	Removal of circulating antibodies	Line insertion related e.g. bleeding, pneumothorax. Air embolism, thrombosis, volume depletion/ overload, metabolic

Table 1.6. Common treatments of myasthenia gravis- Abs = antibodies; PML = Progressive Multifocal Leukoencephalopathy. Nb. most steroid sparing agents increase the risk of opportunistic infection, and can result in reactivation of latent infection e.g. tuberculosis, hepatitis B.

Post intervention status	
Complete stable remission (CSR)	No symptoms/signs for at least 1 yr. Isolated weakness of eyelid closure accepted
Pharmacologic remission (PR)	As per CSR but on MG treatment. Excludes cholinesterase inhibitors
Minimal manifestations (MM)*	Asymptomatic non-functionally limiting weakness, detectable only by careful examination
Improved	Significant improvement in pre-treatment clinical status or reduction of MG treatment e.g. prednisone
Unchanged	No significant change in clinical status or reduction of MG medications
Worse	Substantial worsening of clinical status or increase in MG medications
Exacerbation	Deterioration in clinical findings greater than above criteria, previously CSR/PR/ MM
Died of MG	Death from MG, complications of MG treatment, or within 30 days following thymectomy

Table 1.7. Postintervention status (PIS). The outcome of myasthenia gravis following treatment is typically assessed using the PIS classification.

Disease severity scores improved in both EOMG and LOMG groups (LOMG $\geq$ 40yrs), but a steroid sparing effect was only seen in the EOMG group (Wolfe et al. 2016).

1.8 AChR antibodies

The involvement of AChR-Abs in MG was first systematically demonstrated using radioimmunoassays (RIAs; Lindstrom et al. 1976). This involved the labelling of AChRs with I^{125} bungarotoxin, followed by immunoprecipitation of positive sera using anti-human IgG. The resultant radioactivity was calculated after subtraction of background radioactivity with healthy control samples (**Figure 1.4**). AChR-Abs assessed with this method are present in approximately 80% of patients with generalised MG and 50% with ocular MG (Vincent 2014). Most EOMG patients initially have ocular symptoms that progress to generalised muscle involvement. As mentioned earlier, thymic hyperplasia is frequently detected, and plasma cells contained within germinal centres can spontaneously produce AChR-Abs in culture (Vincent et al. 1978). AChR-Ab titres are heterogeneous in subclass and AChR epitope specificity (Tindall 1981; Demaine et al. 1992; Oosterhuis et al 1983; Andrews et al. 1994). However, many AChR-Abs bind to a region formed by the overlap of the two α -subunits, termed the main immunogenic region (Tzartos et al. 1998; S. Tzartos et al. 1983; Lindstrom et al. 2008). AChR-Ab titres as a whole correlate poorly in grouped analyses of patient disease severity, but has on occasion been noted (Compston et al. 1980). In individual patients, titres have been observed to drop following immunotherapy and thymectomy (Kuks et al. 1991; Newsom-Davis & Vincent 1979). AChR-Abs are predominantly of the IgG1 and IgG3 subclasses and measurement of IgG1 AChR-Ab titres (but not other IgG subclasses) mirrors disease activity in individual patients more closely (Rødgaard et al. 1987). In general AChR-Ab titres gradually decline and are lower in older

patients (Grob et al. 2008; Compston et al. 1980). The exception to this, as previously mentioned, is patients with thymoma, for whom the risk of development is higher in older age groups (Compston et al. 1980).

The evidence supporting the pathogenic role of AChR-Abs is robust. AChR-Abs are capable of binding complement and activating the membrane attack complex (Chamberlain-Banoub et al. 2006; Engel et al. 1977), crosslinking and increasing AChR internalisation (by a factor of 2-3x; Loutrari et al. 1992; Losen et al. 2008; Heinemann et al. 1977; Drachman et al. 1978; Gomez et al. 2010), sterically inhibiting ACh binding to AChRs (Gomez & Richman 1983; Almon et al. 1974; Vernet-der Garabedian 1986; Whiting et al. 1983; Hara et al. 1993), and inhibiting sodium entry (Lang et al. 1988). Of note, however, functional blocking AChR-Abs account for only a small proportion of the AChR-Ab repertoire and it is generally accepted that complement activation and internalisation are the predominant pathogenic mechanisms by which AChR-Abs disrupt neuromuscular transmission. Finally following plasma exchange, patients improve as AChR titres decline and deteriorate following their rise (Newsom-Davis et al. 1978).

1.9 MuSK antibodies

MuSK-Abs, like AChR-Abs are typically detected by RIA (**Figure 1.4**). Purified recombinant I¹²⁵ labelled MuSK is incubated with patient sera, immunoprecipitated with anti-human IgG, and the resultant radioactivity calculated after subtraction of background radioactivity with healthy control sera.

MuSK-Abs in MG sera negative for AChR-Abs was first reported in 2001 (Hoch et al. 2001). Sera immunoprecipitated a 110 kDa protein from MuSK transfected COS7 cells and in mouse C2C12 myotubes alike. They also bound to COS7 cells transfected with the extracellular but not transmembrane/cytoplasmic domains of MuSK. Patient IgG-Abs bound strongly to N-terminal Ig1-4 and Ig1-2 domains, and later studies confirmed the first

IgG1 like domain of MuSK as the major antigenic target of MuSK-Abs (McConville et al. 2004; Huijbers et al. 2016; Ohta et al. 2007).

Pre-incubation with the complete IgG1-like domain, but not 20-mer oligonucleotides covering this region, inhibited binding of MuSK antibodies to full length recombinant MuSK in a competition ELISA, demonstrating the importance of the conformational structure for MuSK-Abs recognition (Huijbers et al. 2013). Epitope mapping also demonstrated conserved specificity for the first Ig-like domain in serial samples from MuSK-MG patients over a 5-year period (Huijbers et al. 2016). Although the pathogenicity of MuSK-IgG had been demonstrated *in vitro*, the observation that MuSK-Abs were mainly IgG4-Abs was surprising as this subclass had traditionally been viewed as anti-inflammatory (McConville et al. 2004; Ohta et al. 2007; Niks et al. 2008; Lindstrom 2004; van der Neut Kolfschoten et al. 2007). This idea stemmed from the observations that IgG4 subclass antibodies are monovalent, non-opsonising, may displace binding of IgG1 or IgE antibodies, and bind to C1q and Fcγ receptors poorly (Nirula et al. 2011). In addition, biopsies from the intercostal and biceps brachii muscles of MuSK-MG patients had not shown significant AChR loss (Shiraishi et al. 2005). However it had been recognised that whilst there were important clinical differences, MuSK-MG was not clinically distinct from AChR-MG. Furthermore clinical fluctuations correlated with MuSK-IgG4 levels, improvement following plasma exchange had been noted in seronegative MG patients (subsequently confirmed to have MuSK-Abs), and maternal to foetal transmission had been reported (Niks et al. 2008; Mossman et al. 1986; Burges et al. 1994; Béhin et al. 2008). Although the clinical evidence favoured a pathogenic role of MuSK-Abs, active and passive transfer studies were required to fulfil Witebsky's revised postulates.

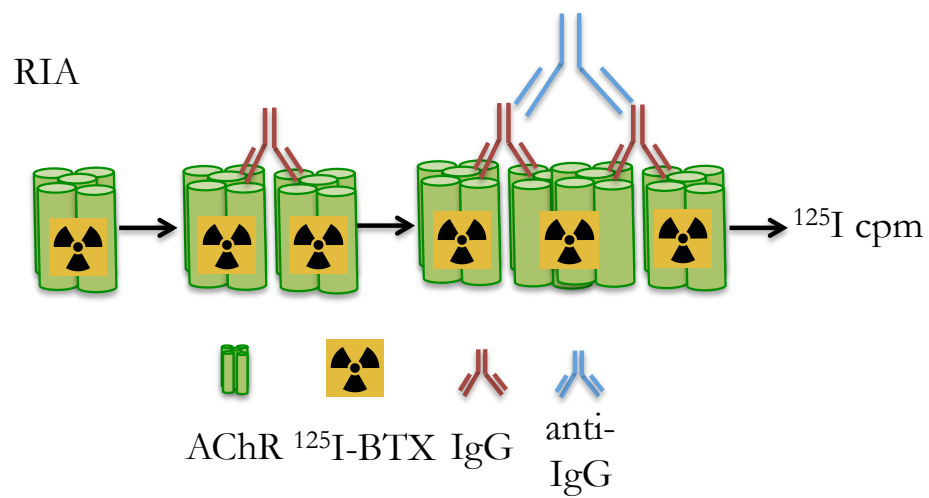


Figure 1.4. AChR radiimmunoassay. Acetylcholine receptors labelled with radioiodine are incubated with patient IgGs (red). Bound IgGs are subsequently immunoprecipitated with a secondary anti-human IgG antibody. The pellet is then washed and resultant radioactivity calculated following adjustment for background levels with healthy control seras.

1.10 MuSK-MG animal models

1.10.1 Active immunisation models

Immunisation against the MuSK ectodomain resulted in muscle weakness in rabbits and mice respectively (Shigemoto et al. 2006; Shigemoto et al. 2008; Jha et al. 2006; Viegas et al. 2012; Mori et al. 2012). Reductions in post-synaptic AChR cluster size and density were noted, and sera from the immunized animals inhibited agrin dependent AChR clustering in myotubes. Importantly, given the predominance of IgG4-Abs in human disease, experimental autoimmune MuSK-MG (EAMG) could be induced in complement-deficient mice. Mori et al. also showed that MuSK-IgG and respective Fab fragments from the immunised mice similarly reduced agrin dependent AChR clustering in myotubes (Mori, Kishi, et al. 2012). This suggested that MuSK-Ab pathogenicity was not premised on the ability to internalise receptors or activate complement, in contrast to AChR-Abs. Intriguingly electrophysiological studies demonstrated a reduction in MEPP/EPP amplitudes and quantal content suggesting both pre- and post-synaptic dysfunction. This contrasted with previous electrophysiological studies in AChR immunised mice where a compensatory increase in quantal content had been consistently observed (Viegas et al. 2012; Plomp et al. 1995).

To determine why there was a predilection for certain muscles to be involved in MuSK-MG, Punga et al. systematically examined different muscles of actively immunised mice and found that postsynaptic fragmentation and reductions in pre-synaptic terminal area were more severe in thoracic, paraspinal, sternomastoid, and masseter muscles compared to leg muscles (Punga et al. 2011). Lower transcript levels of MuSK were found in mouse masseter muscles and it was concluded that this may explain an increased susceptibility to MuSK-Ab effects. However, another study found five-fold higher MuSK protein expression in the masseter as compared to gastrocnemius muscle (Viegas et al. 2012).

Interestingly IgG1 (the IgG4 human analogue) knock-out and wild-type C57BL/6 mice developed MuSK EAMG of a similar clinical severity, suggesting that MuSK ‘IgG4-Abs’ were dispensable for an active MuSK EAMG model (Küçükderden et al. 2016). However, in contrast to human disease, complement deposition was detected at muscle endplates of both sets of mice. It is recognised that active immunisation results in the production of monospecific divalent antibodies that can internalise MuSK limiting the parallels that can be drawn with human disease. In addition the epitopes of the MuSK-Abs produced in that study were unclear.

1.10.2 Passive transfer models

Passive transfer models, though only able to assess the effector functions of antibodies, are not limited by these factors. Several studies confirmed that injection of MuSK-IgG resulted in muscle weakness and CMAP decrement on repetitive nerve stimulation. In some of these studies MuSK-Abs were only retrospectively detected (Mossman et al. 1986; Burges et al. 1994). *Ex vivo* MEPP/EPP amplitude reduction was seen and neither reduction or a compensatory increase in quantal content were clearly observed (Viegas et al. 2012; Morsch et al. 2012). Alterations in nerve terminal and post-synaptic immunostaining were noted as well as a reduction in phosphorylated Src and AChR- β , which are downstream markers of MuSK activation (Cole et al. 2008; Cole et al. 2010; Ghazanfari et al. 2014).

Whilst these studies confirmed the pathogenic role of MuSK-IgG, the confirmation of MuSK-IgG4 Abs pathogenicity required further study. Kloosters et al. subsequently showed that passive transfer of the purified IgG4 fraction from MuSK-MG patients resulted in muscle weakness with electrophysiological evidence of pre- and post-synaptic dysfunction in mice (Klooster et al. 2012). Similar findings were not seen with the IgG1-3 fraction but this may have been due to the use of equivalent protein rather than equivalent MuSK-Abs

concentrations, as the latter are much higher in IgG4 fractions than in the IgG1-3 fractions (Koneczny et al. 2013; Koneczny et al. 2016).

1.11 Mechanism of action of MuSK-IgG4 antibodies

Many *in vitro* studies have utilised cultured myotubes such as the C2C12 cell line, which express neuromuscular junction proteins and provide a useful platform for assessing effector functions of MG autoantibodies. Such studies have shown that IgG4 subclass fractions from MuSK-MG patients disrupt the interaction between MuSK and LRP4 and inhibit agrin dependent and independent AChR clustering (Koneczny et al. 2013; Huijbers et al. 2013; Otsuka et al. 2015; Hoch et al. 2001). In addition, IgG4 but not IgG1-3 fractions from two patients reduced MuSK phosphorylation (Huijbers et al. 2013). Importantly papain digestion of the IgG4 fraction from MuSK-MG sera did not attenuate the inhibition of AChR clustering confirming that divalency was not required for pathogenicity. Furthermore, the majority of MuSK-IgG4 *in vivo* is monovalent and can undergo Fab arm exchange with IgG4 fractions from healthy controls without compromising *in vitro* pathogenicity (Koneczny et al. 2016).

1.12 MuSK-IgG3 antibodies

Whilst it was the pathogenic role of MuSK-IgG4 that was initially questioned, similar questions now apply to MuSK-IgG1-3. Cole et al. reported MuSK internalisation in transfected C2C12 myoblasts after 80 minutes (Cole et al. 2010). Previous studies from this group also reported a reduction post-synaptic MuSK immunostaining *in vivo* following 2 and 14 days, but not 24 hours of daily MuSK-IgG injections (Cole et al. 2010; Ghazanfari et al. 2014; Ghazanfari et al. 2015). However studies using transfected HEK and 3T3 cells have not confirmed these results (Huijbers et al. 2013; Koneczny et al. 2013). A further study

failed to detect a reduction in post-synaptic MuSK immunostaining despite a reduction in AChR density after 5 days of MuSK immunisation (Viegas et al. 2012).

MuSK-IgG1-3 Abs are frequently detected albeit at lower levels than MuSK-IgG4 Abs in MuSK-MG (Leite et al. 2008; Koneczny et al. 2013). Interestingly, MuSK-MG sera incubated with MuSK transfected HEK cells had evidence of C3 deposition and approximately 80% of these sera contained MuSK-IgG1 Abs. This contrasts with muscle biopsy specimens of MuSK-MG patients, which were devoid of complement deposition, though were taken from less clinically affected muscles (Shiraishi et al. 2005). In addition the clinical severity of EAMG in complement deficient and wild type mice is similar (Mori et al. 2012), suggesting that if the complement system does play a role in MuSK-MG, its contribution is likely to be small. On the other hand mice do not readily activate complement.

MuSK-IgG from actively immunised rabbits, but not the respective Fab fragments, can induce agrin independent MuSK phosphorylation and AChR clustering. Similar findings were also observed with MuSK-IgG derived from MuSK-MG patients (Hopf & Hoch 1998; Hoch et al. 2001; Mori et al. 2012; Koneczny et al. 2013). MuSK-IgG1-3 antibodies are divalent and could activate MuSK and downstream pathways to explain these observations. This would in theory promote NMJ stability IgG1-3 fractions from MuSK-MG sera inhibit agrin dependent and independent AChR clustering, suggesting MuSK activation is impaired by an as yet undetermined mechanism (Koneczny et al. 2013).

1.13 MuSK-MG clinical features

Ultimately despite both causing MG, MuSK-MG differs to AChR-MG in epidemiology, genetic associations, pathophysiology, clinical phenotype and treatment responses. A female preponderance is more pronounced in MuSK-MG. The peak age of incidence is in the fourth decade and, unlike AChR-MG a late age peak is not seen. The incidence and

prevalence of MuSK-Abs varies depending on geographical location. In Asian countries such as China the detection of MuSK-Abs in patients negative for AChR-Abs is 4% but in Italy figures of 47.4% have been reported (Yeh et al. 2004; Evoli et al. 2003; Zhang et al. 2007). In the northern hemisphere, prevalence broadly correlates with distance from the equator suggesting that there may be environmental or genetic factors involved in these regions (Vincent et al. 2008). Interestingly higher rates of disease have been reported in patients of African ancestry living in the United States (Guptill et al. 2011; Oh et al. 2009). Juvenile MG patients of African ancestry are susceptible to treatment resistant ophthalmoplegia and ptosis, associated with a polymorphism (387C>T)TGFB1 in transforming growth factor beta-1 (Nel et al. 2015). It may be that there is also a genetic susceptibility that explains the higher prevalence of MuSK-Abs in these populations but this has not been formally studied. In genetic studies of MuSK-MG patients of Dutch and Italian ancestry an association with the major histocompatibility class II DQ5 allele has been identified (Bartoccioni et al. 2009; Niks et al. 2006).

Oropharyngeal involvement with frequent respiratory crises are characteristic, but presentations indistinguishable from non-MuSK-MG, and neck extensor with respiratory muscle weakness are also recognised patterns. Limb involvement, in contrast to AChR-MG is less common (Evoli et al. 2003). Thymic hyperplasia and thymoma also occurs less frequently in MuSK-MG, thymectomy is generally considered unhelpful, and MuSK-Ab titres, unlike AChR-Ab titres, do not fall following thymectomy (Lan et al. 2004; Lauriola et al. 2005; Ponseti et al. 2009; Leite et al. 2005; Guptill et al. 2011; Evoli et al. 2003; Bartoccioni et al. 2006; Evoli et al. 2008). Although there are cases of improvement following thymectomy, the reported patients were also on other therapies, and it is unclear how much these contributed to the observed effects (Saka et al. 2005; Lavernic et al. 2005).

AChEIs are often ineffective, poorly tolerated and may worsen symptoms, which can be useful clinical clues to the serological diagnosis (Guptill et al. 2011; Evoli et al. 2008). The disease course is often severe in the initial years with frequent respiratory crises and

immunotherapy is required in most cases (Evoli et al. 2003; Guptill et al. 2011). Tongue atrophy is a characteristic clinical feature seen in a proportion of MuSK-MG patients, and correlates with the duration of steroid treatment (Farrugia et al. 2006). Magnetic resonance imaging may also demonstrate atrophy of the orbicularis oculi and oris muscles. As with AChR-MG, steroids are highly effective but first line steroid-sparing agents such as azathioprine appear less efficacious (Evoli et al. 2008). Mycophenolate mofetil has been suggested to be a useful alternative, and several studies have reported the beneficial effects of rituximab in refractory disease (Díaz-Manera et al. 2012; Keung et al. 2013; Evoli et al. 2008). As mentioned previously, focal atrophy of facial and tongue muscles can occur and is generally considered to be resistant to treatment.

1.14 Seronegative myasthenia gravis

The term seronegative MG had traditionally applied to those patients who were negative for AChR and MuSK-Abs by radioimmunoassays. However, several lines of evidence suggested that MG without detectable Abs was not an entirely different disease; a) the clinical features were not dissimilar to seropositive MG, b) plasma exchange resulted in clinical improvement, c) maternal to foetal transfer had been reported and d) passive transfer of seronegative sera in animals resulted in MG (Evoli et al. 2003; Sanders et al. 2003; McConville et al. 2004; Lavernic et al. 2005; Romi et al. 2005; Burges et al. 1994; Townsel et al. 2016; Mossman et al. 1986). A proportion of SNMG was also noted to be similar to AChR-MG in terms of thymic histology (Lauriola et al. 2005; Leite et al. 2005). These observations suggested the presence of a pathogenic antibody in seronegative MG that was beyond the limits of detection with traditional RIAs available at the time. This was plausible as it was recognised that antigenic determinants of AChRs for instance could be lost in the solution phase of RIAs.

1.15 Clustered-AChR antibodies by cell-based assays

Subsequently cell-based assays (CBAs) using human embryonic cells transfected with recombinant AChR subunits and rapsyn (which clusters AChRs as they are *in vivo*), allowed for the detection of AChR-Abs in 66% of SNMG patients as well as 50% of patients with previous seronegative ocular MG (**Figure 1.5**; Leite et al. 2008; Jacob et al. 2012). The majority of these Abs were of the IgG1 subclass. Interestingly one of these sera previously thought to be seronegative, had inhibited AChR currents in CN21 cells suggesting a ‘blocking’ effect on AChR function (Spreadbury et al. 2005). However, the remaining sera were concluded to act mainly through complement-mediated mechanisms based on a lack of specificity for the alpha-bungaratoxin site (also the ACh binding site) and inability to reduce surface AChRs in culture. Jacob et al. confirmed these findings by demonstrating that passive transfer of clustered AChR-Abs resulted in reduced MEPP amplitudes and complement deposition at endplates, though the mice did not develop detectable weakness (Jacob et al. 2012). In a further study, clustered AChR-Abs were detected in 16% of patients negative by radioimmunassays (Devic et al. 2014). Although the phenotype of MG with clustered AChR-Abs was similar to AChR-MG, clustered AChR-Abs were more frequently seen in younger patients with ocular disease and high rates of remission (Rodriguez Cruz et al. 2015). Thus the term seronegative MG now needed to account for patients negative for clustered AChR-Abs by CBAs.

1.16 MuSK-Abs by CBA

Using MuSK expressed in HEK cells Leite et al. also detected MuSK-IgG Abs in 8/24 (33%) SNMG sera (Leite et al. 2008). Following this a large multinational European study reported MuSK-Ab detection in 83/633 (13.1%) SNMG samples. Many of these samples were negative for IgG-Abs but positive for MuSK-IgM-Abs (Tsonis et al. 2015). This was of interest as non-IgG fractions from SNMG sera had been shown to inhibit AChR dependent

$^{22}\text{N}^+$ flux and AChR currents in TE671 cells (Barrett-Jolley et al. 1994; Yamamoto et al. 2004). The effect was thought to be in part, dependent upon increased AChR phosphorylation, though additional studies were never conclusive (Plested et al. 2002). Although further validation was required it appeared that CBAs could detect MuSK-Abs in previously SNMG sera.

1.17 LRP4-Abs by CBA

In 2011-2012, 3 studies reported the presence of LRP4-Abs in SNMG, with rates varying between 3-54% (Pevzner et al. 2012; Higuchi et al. 2011; Zhang 2012; Zisimopoulou et al. 2014). The wide range in detection rates may have been due to the use of different assays (Luciferase-reported immunoprecipitation, CBA, and ELISA) and sources of LRP4 (mouse, rat, and human; Higuchi et al. 2011; Zhang et al. 2012; Pevzner et al. 2012). Of the 9 patients described by Higuchi et al. similarities with MuSK-MG were noticeable. Eight had an MGFA>2 and all had generalised disease. An early aggressive course with severe limb weakness and/or progressive bulbar palsy was characteristic and thymic abnormalities were not noted in any of the patients. Notably some of the patients also had MuSK-Abs.

LRP4-Abs were predominantly IgG1-Abs and could inhibit the interaction between LRP4 and agrin (Higuchi et al. 2011). Mice immunised with the LRP4 ectodomain produced LRP4-Abs and developed muscle weakness. NMJ morphology was disturbed and electrophysiological evidence of pre- and post-synaptic dysfunction was observed. Sera from these mice reduced surface LRP4 levels and inhibited agrin dependent MuSK phosphorylation and AChR clustering. Finally, passive transfer of LRP4-Ab containing rabbit sera to naïve mice resulted in similar effects (Shen et al. 2013).

Passive transfer experiments to assess the effector mechanisms of LRP4-Abs from patients have, however, not yet been performed. More recently other studies have detected LRP4-Abs in patients with milder disease including in OMG (Zisimopoulou et al. 2014). In

addition LRP4-Abs have been reported in other diseases, most notably amyotrophic lateral sclerosis (Tzartos et al. 2013; Rivner et al. 2017; Takahashi et al. 2016). It maybe that the LPR4-Abs are produced as a consequence of the degenerative process, analogous to the detection of NMDAR-Abs in sporadic Creutzfeldt-Jakob disease (<5%; Rossi et al. 2015), but further studies are required to confirm this surprising result and determine contribution to disease pathophysiology.

1.18 Other antigenic targets

Three studies have reported the detection of agrin-Abs in SNMG (Cossins et al. 2012; Zhang et al. 2014; Gasperi et al. 2014). Zhang et al. found that agrin-Ab positive sera reduced MuSK phosphorylation and agrin induced AChR clustering (Zhang et al. 2014) but it was not clear if the sera used contained Abs to other known targets. Indeed the majority of agrin-Ab positive sera in both studies also contained AChR-Abs, MuSK-Abs, or LRP4-Abs, therefore the pathogenic contribution of agrin-Abs requires further study.

Collagen-Q (Col-Q) anchors acetylcholinesterase in the extracellular matrix and mutations can result in congenital myasthenia gravis (Rotundo 2003; Donger et al. 1998). A preliminary report detected Col-Q-Abs in 6/161 (4%) of MG patients, but control data were not given. Interestingly two of these patients also had MuSK-Abs but clinical information regarding cholinesterase inhibitor responses were not reported (Cossins et al. 2012). Zoltowska et al. subsequently identified ColQ-Abs in 3% of MG sera but 10% of epilepsy control patients were also positive, therefore the specificity of detection and clinical relevance of these antibodies is unclear (Zoltowska et al. 2015).

Other antigens of note include the intracellular protein titin and the ryanodine receptor (Aarli et al. 1990; Mygland et al. 1992). Though unlikely to be pathogenic given their subcellular localisation, their detection can be a useful marker in MG associated thymoma, and suggests a more severe phenotype in LOMG (Aarli et al. 1998). Antibodies

against cortactin have also been reported in MG but similar proportions were also found in other autoimmune disease sera (19.7% vs. 12.5%; Gallardo et al. 2014)

1.19 Neuromuscular Junction

A neuromuscular junction (NMJ) consists of a pre-synaptic nerve terminal, the synaptic cleft, and a post-synaptic muscle endplate (**Figure 1.2**) The neurotransmitter acetylcholine (ACh), which is stored in pre-synaptic vesicles, upon release traverses the synaptic cleft ($\approx 50\text{nm}$) and binds to ligand-gated fast-acting acetylcholine receptors (AChRs). The pre-synaptic vesicles are precisely aligned opposite the peaks of the folded post-synaptic membrane where AChRs are densely clustered. When stimulated by an action potential, pre-synaptic P/Q-type calcium channels open resulting in an influx of calcium, causing exocytosis of ACh from vesicles into the synaptic cleft facilitating neurotransmission. ACh exocytosis is also dependent upon the local concentration of calcium and fusion of the vesicles to the presynaptic membrane through SNAP proteins (Söllner et al. 1993; Schiavo et al. 1997).

If sufficiently stimulated beyond the threshold potential, voltage-gated sodium channels that lie in the troughs or peri-junctional zones of the post-synaptic membrane open resulting in an end plate potential (EPP). L-type calcium channels, mechanically gated to ryanodine receptors subsequently release calcium leading to excitation-contraction coupling (Sandow 1952). To ensure propagation of an action potential, the EPP typically exceeds the threshold potential by a margin that is termed the ‘safety factor’ (Wood & R Slater 2001). Periodically, pre-synaptic vesicles also spontaneously exocytose their contents, releasing of a ‘quantum’ of approximately 10,000 ACh molecules. These events can be recorded electrophysiologically as a miniature end plate potential (MEPP), a reflection of the post-synaptic AChR density (Katz & Miledi 1965; Fatt & Katz 1952). An EPP consists of approximately 30 quanta, equivalent to a quantal content of 3×10^5 ACh molecules. Following an EPP, ACh dissociates from AChRs and is hydrolysed by acetylcholinesterase

AChE). The P/Q-type calcium channels close, and an outflow of potassium through voltage-gated potassium channels restores the pre-synaptic resting potential in preparation for the next action potential. The post-synaptic density of AChRs is critical to the efficiency of neuromuscular transmission; therefore, it is understandable that a loss of receptor density through mechanisms such as receptor internalisation, complement-mediated damage, dispersal or functional inhibition can result in muscle weakness.

1.20 Development and maintenance of the neuromuscular junction

NMJ development involves the ‘pre-patterning’ of aneural clusters in the central portion of the muscle fibre and guidance of motor axon to these regions for innervation (Wu et al. 2010). Upon innervation by an α -motor neuron the synaptic AChRs enlarge and extra-synaptic AChRs dissipate. A combination of stimulatory and inhibitory factors precisely influence the spatial localisation and density of AChRs. These include inhibitory effects mediated by ACh and a stimulatory signalling pathway involving agrin, low density lipoprotein receptor-related protein 4 (LRP4), muscle specific kinase (MuSK), and docking protein-7 (DOK-7), referred to hence forth as the agrin/LRP4/MuSK, DOK7 (ALMD) pathway.

When ACh activates AChRs, AChR expression and clustering are suppressed and AChR clusters dispersed (Fu et al. 2005; Lin et al. 2005; Wu et al. 2010; Brandon et al. 2003; Tang et al. 2001; Salpeter et al. 1986; Misgeld et al. 2002). Conversely the ALMD pathway promotes AChR clustering, although the precise signalling network downstream of MuSK and DOK7 is not fully understood. Ultimately the ALMD pathway influences the insertion, tethering, and clustering of AChRs to the post-synaptic membrane through the subsynaptic cytoskeletal complex. A hypothetical schematic of AChR clustering and dispersal pathways is presented in **Figure 1.7**.

1.21 The agrin/LRP4/MuSK/DOK7 pathway

1.21.1 Agrin

Agrin, a heparin sulphate proteoglycan, is considered to be the main nerve derived organiser of post-synaptic NMJ differentiation (McMahan 1990). It is produced in the motor neuron, transported axonally and released into the synaptic basal lamina where it is stably maintained. Agrin was originally identified from the electric organ of *Torpedo californica* and was noted to cause aggregation of AChRs on cultured myotubes (Godfrey et al. 1984; Nitkin et al. 1987). Structurally it is composed of an N-terminal basal membrane-binding domain, nine follistatin-like repeats, two laminin-B-like modules, and three laminin globular (LG) domains at the C-terminus (**Figure 1.6**; left). The C-terminal LG domains are critical for AChR clustering and induce cluster formation as effectively as whole agrin (Ferns et al. 1993). Multiple isoforms are generated through alternative splicing, allowing for differing biological activities in the many tissue types agrin is expressed in (Hoch et al. 1993). Neuronal agrin differs by an eight amino acid insertion (Z8) between the second and third LG domains, conferring a 10,000 fold increase in potency to induce AChR clusters (Ferns et al. 1993). Agrin is indispensable to development of the neuromuscular junction and knock-out mice have generalised weakness, are unable to breathe, and die at birth. Examination of muscle tissues from these mice demonstrate that pre-patterning of AChRs is preserved but there are fewer, smaller, and less dense nerve-associated AChR clusters, indicating defective post-synaptic differentiation (Gautam et al. 1996).

1.21.2 LRP4

Due to its close structural and functional relationship to MuSK, LRP4 was referred to as the myotube specificity association component (MASC) prior to its identification as the ligand for agrin (Zhang et al. 2008; Kim et al. 2008). LRP4 is a member of the low-density

lipoprotein receptor (LDLR) family which is involved in lipid metabolism, cholesterol homeostasis, and Wnt signalling (Weatherbee et al. 2006).

Structurally LRP4 consists of a large extracellular domain that contains eight low-density lipoprotein receptor domain class A (LDL_A) repeats, two epidermal growth factor (EGF)-like domains, and four B-propeller domains, each with a C-terminal EFG-like domain (**Figure 1.6**, middle; Burden et al. 2013). Like agrin, LRP4 is indispensable to survival and LRP4^{-/-} mice die at birth. Pre- and post-synaptic defects are present including loss of pre-patterning, abnormal motor axon growth/branching, absence of post-synaptic AChR clustering and reduced synapse-specific transcription in muscle nuclei (Weatherbee et al. 2006). LRP4 is also involved in retrograde signalling and can bind to motor axons to induce clustering of synaptic-vesicle and active-zone proteins (Yumoto et al. 2012).

1.21.3 MuSK

Like agrin, MuSK was originally identified in *Torpedo californica* (Jennings et al. 1993) and is a type 1 single-pass transmembrane glycoprotein of 110 kDa that colocalises with AChRs at the NMJ (Valenzuela et al. 1995). The extracellular region contains three immunoglobulin-like (Ig) domains and a cysteine-rich domain, the latter of which resembles the CRD of Frizzled proteins. The transmembrane domain consists of a single helix connected to the cytoplasmic juxtamembrane region, followed by a tyrosine kinase domain and a short cytoplasmic tail (**Figure 1.6**; right). MuSK^{-/-} mice are paralysed, unable to breathe, and die shortly after birth. Pre- and post-synaptic differentiation is abnormal with absent pre-patterning and synapse specific AChR gene expression. Synapse formation can be rescued in MuSK^{-/-} mice by expression of the juxtamembrane and kinase region highlighting the critical role of these domains in NMJ development (Herbst et al. 2002). Activation of MuSK, like many other tyrosine kinase receptors, is dependent upon relief of auto-inhibition and dimerisation (Hopf & Hoch 1998). However, as discussed below, MuSK is activated through LRP4 rather than directly. Auto-inhibition of the juxtamembrane domain and the

activation loop, contained within the kinase domain regulates MuSK catalytic activity. The Tyr553 tyrosine residue in the juxtamembrane domain and three other residues (Tyr 750, Tyr 754, Tyr 755) within the activation loop are the major sites of tyrosine phosphorylation (Watty et al. 2000; Till et al. 2002). Full activation requires phosphorylation of Tyr553 and Tyr754/755 within the activation loop (Hubbard & Gnanasambandan 2013). There is a serine phosphorylation site, S751 that also modulates MuSK kinase activity (Camurdanoglu et al. 2016). One study reported that MuSK endocytosis was required for agrin-dependent MuSK activation and AChR clustering (Zhu et al. 2008). However, another study did not find that co-transfection with DOK7 in COS-7 cells (which activates MuSK) enhanced endocytosis, although agrin specific activation was not assessed (Luiskandl et al. 2013).

MuSK activation not only stimulates kinase activity but facilitates recruitment of downstream signalling proteins. The Tyr553 residue is within an NPXY sequence motif that upon phosphorylation recruits Docking Protein 7 (DOK7; Bergamin et al. 2010). MuSK is also required for the post-synaptic anchoring of acetylcholinesterase through a ternary complex that includes perlecan (Cartaud et al. 2004).

1.21.4 DOK7

DOK7 is a cytoplasmic adaptor protein that contains N-terminal pleckstrin homology (PH) and a phosphotyrosine binding (PTB) domain. The DOK7 PTB domain in conjunction with the PH domain is involved in binding to the juxtamembrane region (Tyr553) of MuSK. DOK7 dimerisation facilitates juxtaposition of adjacent MuSK kinase domains resulting in trans-phosphorylation of Tyr750/754/755 and reduction of kinase autoinhibition (please see **Section 1.21.6- agrin/LRP4/MuSK/DOK7 interaction**; Bergamin et al. 2010). Recruitment of DOK7 is necessary for MuSK activation and DOK7^{-/-} mice fail to form neuromuscular junctions and AChR clusters (Okada et al. 2006). DOK7 also contains tyrosine phosphorylation sites, but these are dispensable for MuSK and AChR- β subunit tyrosine phosphorylation (Hallock et al. 2010).

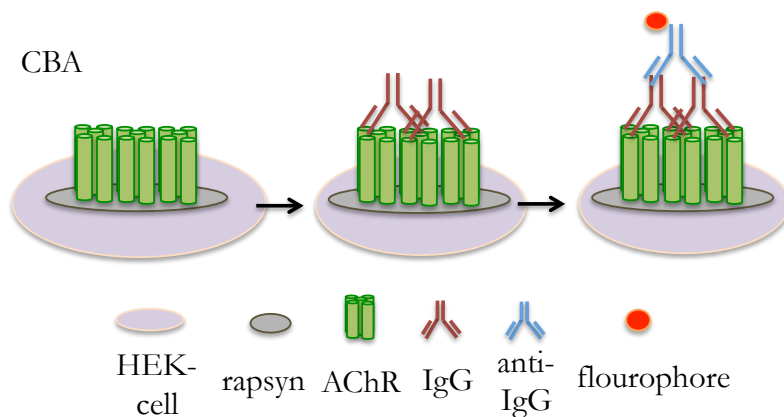


Figure 1.5. Clustered AChR cell-based assay- Human embryonic kidney cells (HEK-cells) are transfected with subunits of the acetylcholine receptor (AChR) and the scaffolding protein rapsyn (tagged with green fluorescent protein), which clusters AChRs as they are *in vivo*. Patient IgGs (red) are then incubated with live transfected HEK cells, and bound IgGs detected with a fluorescent conjugated anti-human IgG secondary antibody. Flourescent microscopy allows for the assessment of colocalisation between transfected HEK cells (green) and labelled patient IgGs (red).

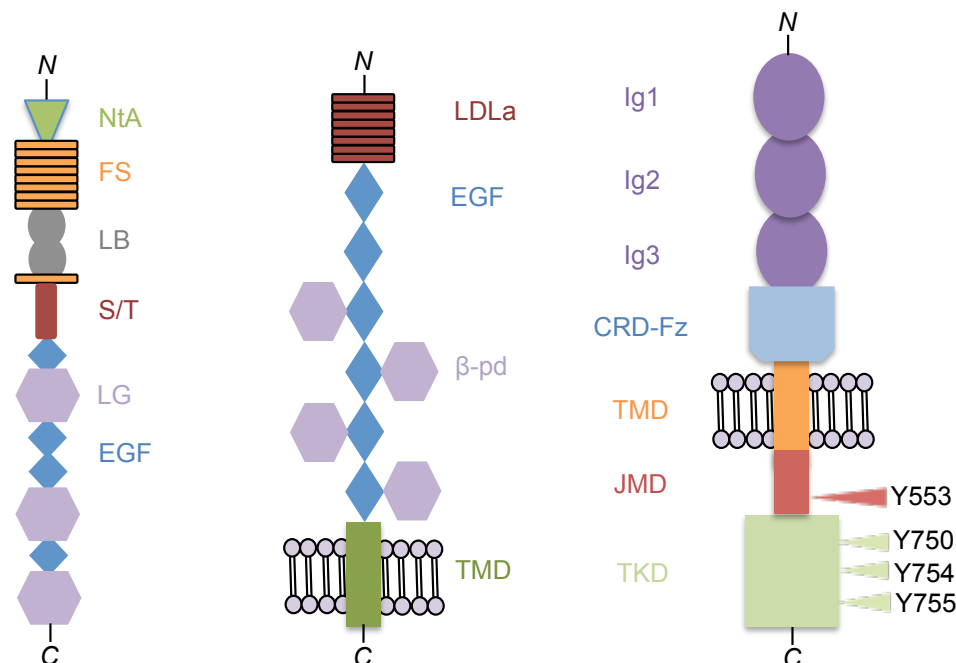


Figure 1.6. *Left*- agrin consists of an amino terminal domain (NtA), nine follistatin-like domains (FS), two laminin-B-like modules (LB), a serine-threonine rich domain (S/T), followed by four EGF domains (EGF), and three laminin globular domains (LG). *Middle*: LRP4 consists of eight LDL class A repeats (LDLa), two EGF-like domains (EGF), followed by four β -propeller domains (β -pd) alternating with four further EGF-like domains, a transmembrane domain (TMD) and a short cytoplasmic tail. *Right*: MuSK consists of three Ig-like domains (Ig1-3), a cysteine rich frizzled domain (CRD-Fz), a transmembrane domain (TMD), a juxtamembrane domain (JMD), a tyrosine kinase domain (TKD), and a short cytoplasmic tail. The Y553, Y750, Y754, Y755 residues are necessary for full activation of MuSK.

1.21.5 Rapsyn

Rapsyn is a scaffolding protein that anchors AChRs through β -dystroglycan and utrophin to F-actin (collectively referred to as the actin cytoskeletal complex; Glass et al. 1996; Moransard et al. 2003; Cartaud et al. 1998; Rybakova et al. 2006; Wang et al. 1999; Phillips et al. 1997). It contains a one coiled-coil domain that binds to AChRs, a seven tandem tetratricopeptide repeat domain that facilitates self-association, and a zinc ring finger domain that interacts with β -dystroglycan (Ramarao et al. 2001; Ramarao & Cohen 1998). The tyrosine Y390 phosphorylation site on the AChR- β subunit regulates rapsyn association, post-synaptic anchoring and increases AChR surface receptor stability (Borges et al. 2008; Rudell & Ferns 2013).

Actin polymerisation is a critical component in AChR formation and indeed rapsyn interacts with alpha-actinin, an F-actin cross-linking protein, in an agrin-dependent manner (Dai et al. 2000; Dobbins et al. 2008). The indispensable nature of rapsyn to NMJ formation has been shown in knock-out mice that are unable to move and die shortly after birth from respiratory failure (Gautam et al. 1995). Analysis of the diaphragm muscles shows preserved pre-patterning but an absence of AChR clusters. In addition increased expression of rapsyn prevents AChR loss and CMAP decrement in a passive transfer AChR-Ab mouse model (Losen et al. 2005).

1.21.6 Agrin, LRP4, MuSK, and DOK7 structural interaction

Unlike most receptor tyrosine kinases MuSK is not activated directly, but through a ligand induced interaction with LRP4. An eight amino acid sequence (z8) contained within the LG3 domain unique to neuronal agrin is required for the interaction with LRP4. The LDLa repeats 6-8, first B-propellor domain and two intervening EGF-like domains of LRP4 are necessary (and sufficient) for the interaction with agrin (Zhang et al. 2011). The agrin-LRP4 interaction is initially binary and dependent on the z8 insert, but subsequently a 2:2

tetrameric complex is formed creating interfaces between agrin-LRP4 and agrin-agrin. The importance of this complex is illustrated through mutations that affect the z8 interface and impair co-immunoprecipitation of agrin-LRP4, MuSK activation, and AChR clustering (Zong et al. 2012).

Agrin also promotes binding between LRP4 and MuSK (Zhang et al. 2011). The first three B-propeller domains of LRP4 are involved in the interaction with the first Ig-like domain of MuSK, an interaction which can occur independently of agrin (Zhang et al. 2011). The interaction involves isoleucine-96, which is within the first Ig-like domain of MuSK. MuSK dimerization involves Methionine-48 and Leucine-83 residues in the first Ig-like domain, and all three of the aforementioned residues are required for agrin induced MuSK activation (Stiegler et al. 2006). LRP4 self-associates, which also likely influences trans-phosphorylation of MuSK (Zhang et al. 2011).

Thus the agrin-LRP4 tetrameric complex promotes MuSK/LRP4 interaction and MuSK dimerisation, and relieves MuSK autoinhibition. This facilitates stepwise phosphorylation of Tyr residues within the juxtamembrane and activation loop. This in turn enables recruitment and dimerization of DOK7 further juxtaposing MuSK kinase domains as well as activating DOK7 kinase activity and mediating downstream signalling.

1.21.7 Positive regulation of AChR clustering

MuSK activation plays a central role in the recruitment of several other downstream mediators of AChR clustering aside from DOK-7. MuSK interacts with Src and Abl kinases, which are involved in signal transduction for early and late agrin induced MuSK and AChR phosphorylation (Mittaud et al. 2004). Inhibition of Abl kinase activity with STI-571 (Gleevec) reduces agrin-dependent MuSK tyrosine phosphorylation and blocks AChR clustering in myotubes. The former suggests that Abl kinases also provide reciprocal positive feedback for MuSK tyrosine phosphorylation.

MuSK interacts with geranylgeranyl transferase (Ggt) to activate GTPases of the Rho family. Inhibition of Ggt or the Rho GTPase family inhibits agrin-dependent AChR clustering in myotubes whilst agrin induces Rac1 activation and the formation of AChR micro-aggregates (Luo et al. 2003; Weston et al. 2000). Rho activation promotes maturation of these AChR microclusters, possibly through the serine/threonine kinase P21-activated kinase (Pak1; Luo et al. 2002; Weston et al. 2003). Pak1 interacts with MuSK through Dishevelled (Dvl) and inhibition of Pak1 or its interaction with Dvl inhibits agrin induced AChR clustering (Luo et al. 2002). Pak1 may also phosphorylate cortactin which through the Arp2/3 complex promotes actin polymerisation (Webb et al. 2006; Weaver et al. 2001). Cortactin tyrosine phosphorylation is increased by agrin. Forced expression of a phospho-inactive mutant or downregulation of cortactin attenuated agrin dependent AChR clustering. Casein kinase 2 (Ck2) associates with and phosphorylates Dvl. Interestingly Ck2-dependent serine phosphorylation of MuSK appears to regulate AChR clustering, and muscle specific Ck2 knockdown results in a myasthenic phenotype *in vivo* (Gao & Chen 2010; Wharton 2003). Dvl also activates Ca^{2+} /calmodulin-dependent protein kinase II (CamKII) and Protein kinase C (Pkc) which have opposing roles in AChR cluster stability. Inhibition of CamKII reduces AChR cycling from the cytosol to the post-synaptic membrane but Pkc accelerates AChR removal and reduces AChR recycling (Martinez-Pena y Valenzuela et al. 2010; Sheldahl et al. 2003). Dvl also interacts with adenomatous polyposis coli (APC) to stabilise microtubules (Matsumoto et al. 2010).

Wnt proteins bind to frizzled receptors and promote NMJ formation in *Drosophila* (Packard et al. 2002; Mathew et al. 2005). As mentioned earlier the cysteine rich domain of MuSK contains a frizzled domain, and in zebrafish Wnt 11r can bind to the MuSK homologue *unplugged* to guide motor axons (Jing et al. 2009). There are 19 Wnt proteins in mice and humans, of which Wnt 9a/9b/10b/11/16 promote AChR clustering, and Wnt3a inhibits AChR clustering. When immobilised on beads Wnt 9a and 11 bound the ectodomains of LRP4 and Wnt 11 bound to the CRD of MuSK. The effects of Wnt9a and

Wnt11 on AChR clustering were dependent on the presence of MuSK and LRP4, and appeared to be mediated through an increase in MuSK dimerisation and phosphorylation (Zhang et al. 2012). Dvl is intrinsically involved in the relay of Wnt signalling pathways. Wnts, through Dvl result in an accumulation of B-catenin, that regulates AChR clustering through interaction with Rapsyn.

The heat shock protein 40, Tumorous imaginal discs 1 (Tid1) binds to the MuSK juxtamembrane and is involved in signalling downstream from MuSK. Short hairpin RNA interference of Tid1 inhibited AChR clustering *in vitro* and disrupted both structure and electrophysiological function of adult mouse neuromuscular synapses. MuSK activation was not affected but co-immunoprecipitation of MuSK/DOK7 in agrin treated myotubes was reduced as well as AChR clustering in DOK7 overexpressing myotubes. Collectively this suggests that Tid1 acts downstream of MuSK and regulates in part the binding of DOK7 to MuSK. TID1 also binds to heat shock cognate protein 70 and may reduce its inhibitory regulation of Rac1, Cdc42, and RhoA (Linnoila et al. 2008).

Caveolin-3 expression promotes agrin dependent AChR clustering that appears dependent upon MuSK and Rac1 phosphorylation. Focal adhesion kinase (Fak) regulates expression of Caveolin-3 and is also implicated in cytoskeletal reorganisation (Quach et al. 2009). Caveolin-3 null mice exhibit some electrophysiological evidence of NMJ dysfunction. Caveolin-3 mutations in humans are associated with a number of diseases including limb girdle muscular dystrophies, myopathies, and rippling muscle disease (Hezel et al. 2010).

Interestingly amyloid precursor protein (APP) can interact with LRP4, and stimulate MuSK phosphorylation, as well as AChR clustering independent of agrin. Agrin potentiates the interaction between LRP4 and APP increasing AChR clustering (Choi et al. 2013).

DOK7 is required for full activation of MuSK, but is in turn also phosphorylated on Tyr396 and Tyr406. This allows for the recruitment of the non-catalytic adaptor proteins Crk and Crk-L (CT10 regulator of kinase) to the c-terminal domain of DOK7 (Hallock et al. 2010). The most common DOK-7 mutation associated with congenital MG

(1124_1127dupTGCC) truncates the DOK-7 C-terminus inhibiting Crk/Crk-L recruitment without affecting MuSK kinase activity (Beeson et al. 2006). The importance of Crk/Crk-L has also been demonstrated through conditional inactivation in the skeletal muscle of mice. This was associated with severe defects in pre- and post-synaptic differentiation and resulted in neonatal lethality likely due to respiratory failure (Hallock et al. 2010). Crk/Crk-L interact with Sorbs 1 and 2 of the vinexin family of adaptor proteins. Knockout of Sorbs 1 and 2 in C2 myotubes attenuated agrin dependent AChR clustering. Though lacking catalytic activity, the SH3 domains of Crk/Crk-L and Sorbs1/2 can bind proteins such as the Abl 1/2 non-receptor tyrosine kinases, which as previously mentioned are involved in AChR clustering and actin dynamics (Finn et al. 2003; Hallock et al. 2015). In addition an interaction between Crk/Crk-L and the Rac/Rho/P21-activated kinase (PAK1) pathway has been suggested (Hallock et al. 2010; Weston et al. 2000; Weston et al. 2003).

Neuregulin-1 is the ligand for ErbB2 and ErbB4 receptors, which are located in the depths of synaptic folds (Zhu et al. 1995; Buonanno & Fischbach 2001). Interestingly mice that are heterozygous for neuregulin-1 exhibit myasthenic symptoms and reduced post-synaptic AChR density (Trinidad et al. 2000; Sandrock et al. 1997). The effect of neuregulin-1 on AChR clustering and disassembly appears to be time dependent. Application of agrin to myotubes induced an LRP4/MuSK dependent peak in extracellular signal-regulated kinases 1 and 2 (Erk1/2) within 5-10 minutes. Activation was abolished in myotubes deficient of either LRP4 or MuSK and inhibition of the Erk 1/2 upstream activator, Mek1/2 potentiated agrin dependent clustering (Rimer 2010). Interestingly Neuregulin-1 is a known activator of Erk 1/2, however Ngo et al reported an increase in agrin dependent AChR clustering in myotubes at 4 hours (Ngo et al. 2012). Presumably in the short term, regardless of Erk 1/2 contribution, the net effect of neuregulin-1 is to promote AChR clustering. The mechanism is unclear but upon activation by Neuregulin-1, the tyrosine phosphatase Src homology 2 domain-containing phosphatase 2 (Shp2) binds to the ErbB complex. This interaction has been suggested to inhibit the phosphatase activity of Shp2, a negative

regulator of MuSK phosphorylation. With longer periods of exposure Neuregulin-1 has a negative effect on AChR clustering. Neuregulin-1 decreased spontaneous and agrin dependent AChR clusters in myotubes and increased dispersal of pre-formed agrin dependent AChR clusters at 23 hours (Trinidad & Cohen 2004; Ngo et al. 2004).

AChR clustering is ultimately dependent upon AChR cycling dynamics, anchorage, and stability at the post-synaptic membrane. The delivery of AChRs to cluster sites, is dependent on capture by microtubules and the microtubule plus-end tracking protein CLASP2 (Cytoplasmic linker associated protein 2). CLASP2 knockout myotubes fail to cluster AChRs in response to agrin, which is rescued by expression of GFP-CLASP2. GSK3 β phosphorylation negatively regulates CLASP2 function. Expression of phospho-resistant CLASP2 rescues agrin-dependent AChR clustering in myotubes expressing a constitutively active GSK3 β . In addition the amount of active CLASP2 influences AChR cluster size (Basu et al. 2014). Interestingly, inhibition of GSK3 β phosphorylation can be mediated by Dvl as demonstrated in mouse neuronal cell cultures (Krylova et al. 2000).

Actin polymerisation is required for AChR cluster formation/stability, and is dependent upon cholesterol/sphingolipid-rich membrane microdomains or membrane rafts triggered by agrin (Cartaud et al. 2011). As previously mentioned, Rapsyn is thought to anchor AChRs to the actin cytoskeleton and this interaction is dependent upon β -catenin, and in turn α -catenin. The interaction between β -catenin and rapsyn is increased by agrin and inhibition of β -catenin attenuates agrin dependent AChR clustering in C2 myotubes. However, simply increasing β -catenin levels can be antagonistic as upregulation in C2C12 myotubes resulted in fewer agrin dependent AChR clusters. It may be that the pro-clustering event is dependent on upregulation of other interacting proteins that occurs normally with agrin (Wang et al. 2008).

Coronin 6, a member of the coronin family of proteins forms a complex with AChRs and actin. Both *in vitro* and *in vivo* knock down impaired stability of AChR clusters suggesting

its importance in clustering AChRs through anchorage to actin (Chen et al. 2014). Interestingly Coronin-6 was also immunoprecipitated with Crk-L and may therefore be involved in DOK7 signalling pathways (Hallock et al. 2015).

In vivo time-lapse imaging has demonstrated the role of the serine/threonine kinase protein kinase A (PKA) on the dynamics of AChR removal and recycling. PKA activation and PKC inhibition prevents removal of AChRs and promotes recycling of internalised AChRs to the postsynaptic membrane. By contrast PKC activation and PKA inhibition accelerates AChR removal and reduces AChR recycling (Martinez-Pena y Valenzuela et al. 2013). Calcium dependent pathways are also involved in AChR phosphorylation, agrin dependent AChR clustering and AChR receptor cycling. AChR enriched membrane fractions of *Torpedo californica* exhibited a 3- to 6-fold increase in endogenous phosphorylation with calmodulin and calcium (H Smilowitz 1981). Additionally fast (but not slow) chelation of calcium inhibited AChR clustering in myotubes (Megeath & Fallon 1998).

Despite many of the post-translational modifications described above involving tyrosine phosphorylation, a time dependent phosphopeptide enriched proteomic analysis of agrin treated C2C12 myotubes demonstrated that tyrosine phosphorylation accounted for only 1-2% of all phosphorylation events. Although early involvement of MuSK Y553 and AChR- β Y390 was detected Dok7, Crk/Crk-L and Src kinases were notably absent. Many of the proteins identified were involved in actin cytoskeleton arrangement and RhoA signalling. Clip2, components of synaptic podosomes (talins, vinculin, paxillin), Pdzrn3, L15 β , Cdk5, CK2 were also identified. Cluster maturation is influenced by L15 β and components of synaptic podosomes and Paxillin is a scaffolding protein that upon tyrosine phosphorylation activates Rac1 (Proszynski & Sanes 2013; Proszynski et al. 2009). Thus it is clear that microtubular and scaffolding dynamics that influence receptor cycling and tethering to the actin cytoskeleton complex play an important role in AChR clustering.

1.21.8 Negative regulation of AChR clustering

Negative regulators of AChR clustering have been postulated to have a role in the spatial and temporal localisation of synaptogenic signalling. Most of the studies identifying these proteins have used *in vitro* myotube models with carbachol, an ACh agonist that disperses AChRs in cultured myotubes.

MuSK phosphorylation is negatively regulated by the ubiquitously expressed cytosolic non-receptor phosphotyrosine phosphatase Shp2. Signal regulatory protein- α stimulates Shp2 activity whilst Shp2 inhibition increases both agrin dependent and independent AChR clusters in C2C12 myotubes (Zhao et al. 2007). Structurally Shp2 consists of two SH2 domains, a catalytic protein tyrosine phosphatase (PTP) domain, a c-terminal tail with tyrosyl phosphorylation sites (Y542 and Y580), and a proline-rich motif. The n-terminal SH2 domain inhibits the catalytic PTP domain in the basal state, but upon stimulation undergoes a conformational change to facilitate activation of the PTP domain.

The serine/threonine kinase, cyclin dependent kinase 5 (Cdk5) is involved in carbachol induced dispersal of non-agrin stabilised AChR clusters, as has been demonstrated in agrin null mice. Agrin/CDK5^{-/-} mice or the inhibition of Cdk5 using roscovitine in agrin^{-/-} mice increased the number of AChR clusters observed at E16.5. In addition carbachol increased Cdk5 kinase activity and dispersed pre-formed agrin dependent AChR clusters, an effect which was inhibited by Roscovitine in C2C12 myotubes (Lin et al. 2005).

Nestin, in concert with p35 is required for Cdk5 activation. In particular the Thr316 site of Nestin is critical to carbachol induced Cdk5 activation and AChR cluster dispersal. Reducing nestin expression in agrin null mice increased the number of AChR clusters along the central band of the diaphragm muscle as compared to control agrin^{-/-} mice (Yang et al. 2011).

Calpains are ubiquitously expressed calcium-activated cysteine proteases that are activated by carbachol. Calpain activation may act in a Cdk5 dependent manner as cleavage

of its substrate p35 yields p25, an activator of Cdk5. Inhibition of either calpain or Cdk5 promotes AChR cluster stability in the presence of carbachol. Disruption of an interaction between rapsyn and calpain also increases carbachol mediated AChR cluster dispersion, suggesting that rapsyn may negatively regulate calpain (Chen et al. 2007).

Caspase-3, better known for its role in apoptosis, has also been implicated in carbachol mediated dispersal of AChR clusters, through a mechanism involving cleavage of Dishevelled (Wang et al. 2014). The Dvl1 cleavage site resides in the DEP (Dishevelled, Egl-10, and Pleckstrin) domain, which is involved in the interaction between MuSK and Dvl1. Carbachol treatment inhibited the coimmunoprecipitation of Dvl1 with MuSK, an effect not seen in Caspase-3^{-/-} myotubes. ACh stimulation also increased cleaved (active) Caspase-3 and dispersed pre-formed agrin-dependent AChR clusters in C2C12 myotubes, an effect which was blocked by inhibition of Caspase-3 or Dvl1 cleavage. Agrin inhibited carbachol mediated Caspase-3 activation and stabilised AChR clusters. This effect was dependent at least in part by, Hsp90 as the protective effect of agrin was negated by either the addition of 17-(allylamino)-17-demethoxygeldanamycin (an inhibitor of heat shock protein 90) or the addition of siHSP90 β in muscle cells.

In summary an increasing number of interacting molecules and pathways have been implicated in AChR clustering/dispersal dynamics. Exactly how these interactions regulate one another to maintain equilibrium between AChR clustering and dispersal remains unclear. It is reasonable to assume that any factor, heritable or acquired that reduces AChR clustering or enhances dispersal could adversely affect neuromuscular transmission and cause muscle weakness. Following this logic, targeted modulation of any factor that increases AChR clustering may be beneficial in MuSK-MG, in which the main pathogenic action appears to be functional inhibition of the agrin/MuSK/LRP4/DOK7 AChR clustering pathway.

1.21.9 Aims

1. a) To determine the prevalence of AChR-, MuSK-, and LRP4-Abs in a large international MG cohort using standard and advanced serological detection methods (radioimmunoassays and cell-based assays)
b) To optimise and validate a MuSK cell-based assay
c) To identify novel antigens in MG samples negative for known candidate antigens.
2. a) To analyse the serological features of a MuSK-MG cohort (n=71; positive by radioimmunoassay) to be used for functional and therapeutic studies.
b) To analyse the differences between patients positive by MuSK radioimmunoassay and cell-based assay
3. a) To assess the effects of IgG1-3 and IgG4 from MuSK-MG patients on MuSK phosphorylation
b) To determine if inhibition of the tyrosine phosphatase, SHP2 increases MuSK phosphorylation and clustering of acetylcholine receptors in the presence of MuSK-MG sera.

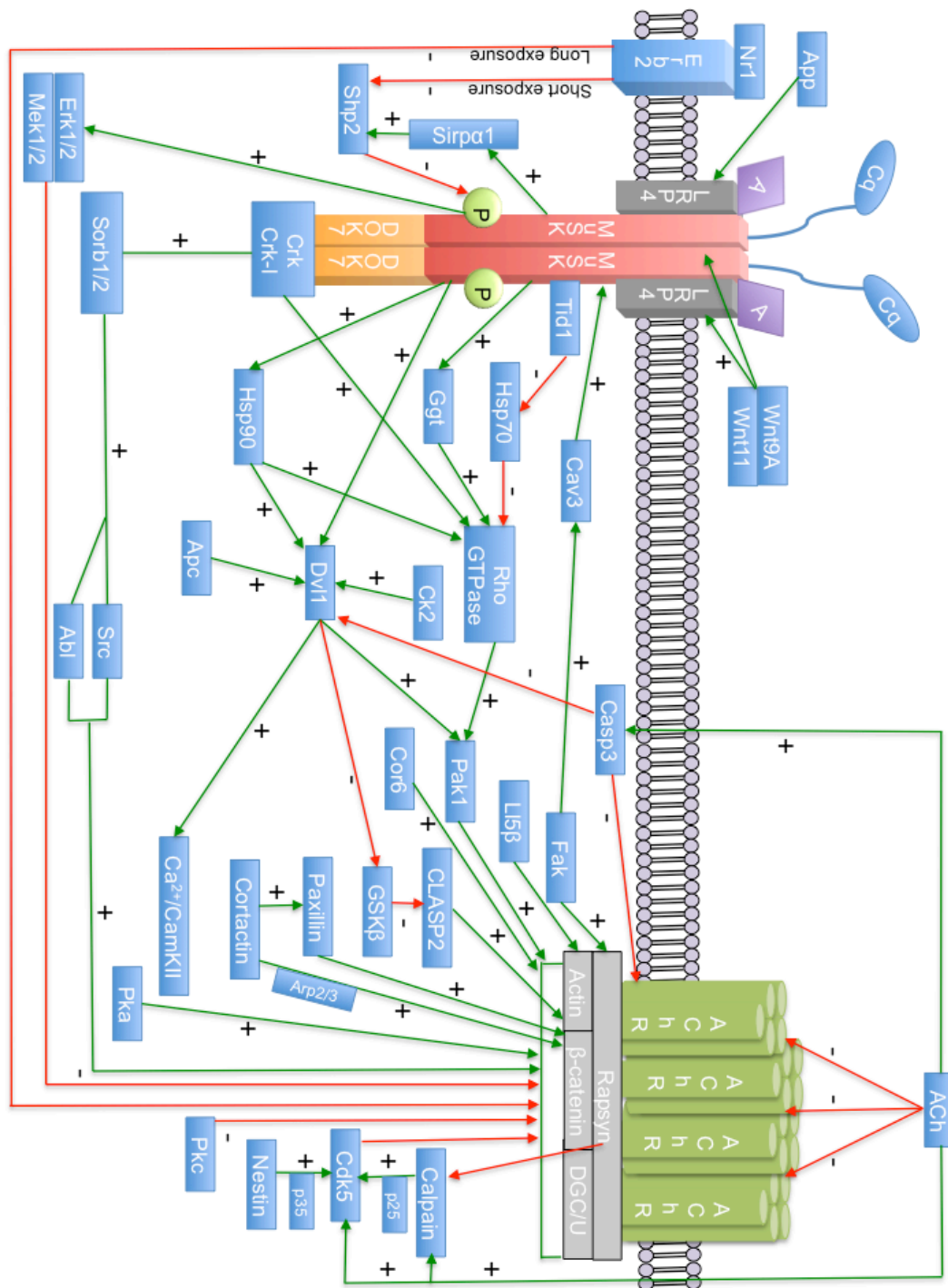


Figure 1.7. Hypothetical schematic of positive and negative regulation of AChR clustering- Cq = collagen-Q; App = amyloid precursor protein; A = agrin; Wnt = wingless; Nr1 = neuregulin-1; LRP4 = low-density lipoprotein receptor-related protein 4; MuSK = muscle specific kinase; Erb2 = epidermal growth factor receptor-2; Tid1 = tumourous imaginal disc 1; P = phosphorylation; Sirp α 1 = signal regulatory protein alpha 1; Shp2 = Src homology 2 domain containing phosphotyrosine phosphatase 2; DOK7 = docking protein 7; Crk-CT10 regulator of kinase; Sorb = sorbin and SH3 domain-containing protein; Erk = extracellular signal-regulated kinase; Cav3 = caveolin-3; Hsp = heat shock protein; Ggt = geranylgeranyl transferase 1; Ck2 = casein kinase 2; Dvl = dishevelled; Apc = adenomatous polyposis coli; Abl = Abelson murine leukaemia viral oncogene homolog 1; ACh = acetylcholine; Casp3 = caspase-3; Fak = focal adhesion kinase; Pak1= p21 activated kinase 1; CLASP2= Cytoplasmic linker associated protein 2; GSK β = glycogen synthase kinase 3 beta; Ca²⁺ = calcium; CamKII = calmodulin-dependent protein kinase II; Pka = protein kinase A; Pkc = protein kinase C; Cdk5 = cyclin-dependent kinase 5; Cor6= Coronin-6; DGC = dystrophin-associated glycoprotein complex; U = utrophin

Chapter 2: Materials and Methods

2.1 Tissue culture

All tissue culture techniques were performed in class II tissue culture hoods under sterile conditions. Human Embryonic Kidney-293 (purchased from ECACC), TE671, and CN21 cells were grown in Dulbeccos's modified Eagle's medium (DMEM) supplemented with 10% FCS (Sigma-Aldrich) and 1% each of Penicillin, Streptomycin, and Amphotericin B (PSA, Invitrogen) at 37°C in an atmosphere of 5% CO₂ (Beeson et al. 1996). C2C12 myoblasts (purchased from Sigma-Aldrich) were grown in growth medium (DMEM supplemented with 15% FCS and 1% PSA) and differentiated for 5-6 days in differentiation medium (DMEM supplemented with 2% FCS and 1% PSA) at 37°C in an atmosphere of 8% CO₂. DOK7 infected C2C12 myoblasts were grown in DMEM with 15% FCS and 0.1% Puromycin at 37°C in an atmosphere of 8% CO₂.

2.2 Materials

Product	Company	Product Number
¹²⁵ I labelled AChR	RSR Ltd	RBA/25
¹²⁵ I labelled MuSK	RSR Ltd	MSK/TR
Alexa Fluor-594 conjugated bungarotoxin	Invitrogen	B13423
Amersham ECL Western blotting system	GE healthcare	RPN2108
Bovine Serum Albumin	Fisher Scientific	12737119
Centrifugal Filer units	Millipore	UFC30GV0S
DMEM	Sigma	D6429-24X500ML
DynaMag™-2 Magnet	Life Technologies	12321D
Fungizone® (antibiotic-antimycotic)	Gibco	15240

HALT phosphatase inhibitor cocktail	Fisher Scientific	15290241
HEPES	Sigma	H3375
MINI Dialysis Device	Fisher Scientific	10668284
NuPage LDS sample buffer	Invitrogen	NP0008
NuPage reducing agent	Invitrogen	NP0004
NuPAGE sample reducing agent	Life Technologies	NP0004
Paraformaldehyde	TAAB Laboratories Equipment Ltd.	F003
Polybrene	Insight Biotechnology Ltd	sc-134220
Protease inhibitor cocktail	Sigma	P8340
Protein G coupled magnetic beads, Dynabeads	Invitrogen	100.03D
Puromycin	Sigma	P9620
RIPA Buffer	Sigma	R0278
SeeBlue® Plus2 Pre-Stained Standard	Life Technologies	LC5925
Streptavidin coupled Dynabeads	Life Technologies	65601
VisionWorks®LS Image Acquisition Software	UVP	97-0186-03
NSC87877 (SHP2 inhibitor)	Tocris	NSC87877

Table 2.1. General materials used-.

Antibody	Company	Product No	Species	Application	Dilution
Anti-MuSK polyclonal	R+D systems	AF562	goat	IP/WB	1:100
Anti-MuSK polyclonal	Abcam	AB5619	mouse	IP	1:100
Anti-human IgG Alexa Fluor 568	Invitrogen	A21090	goat	CBA	1:750,
Anti-human IgG Alexa Fluor 488	Invitrogen	A11013	goat	CBA	1:750
Anti-human IgG Fc(γ)	Fisher Scientific	31125	goat	CBA	1:750
HRP-conjugated anti-mouse	Dako	P044701-2	goat	WB	1:2000
HRP-conjugated anti-goat	Dako	P044901-2	rabbit	WB	1:2000
Anti-human IgG1/2/4	Sigma	I2513 I5635 I7260 I7385	mouse	CBA	1:50
Anti-goat IgG Alexa Fluor 568	Fisher Scientific	A11079	rabbit	CBA	1:750
Anti-human IgM	Invitrogen	A21216	goat	CBA	1:750

Anti-Phosphotyrosine Antibody	Millipore	05-321	mouse	WB	1:500-1:800
Anti-Mab 124	Dr. Tzartos	Gift	mouse	WB	1:1000

Table 2.2. Antibodies used.

2.3 Serum samples

MG sera=250 were collected from archived and new cohorts (Germany=10, Turkey=16, Japan=8, South Korea=13, Philippines=12, UK=71, Norway=56, Italy (Rome)=25, South Africa=39). MuSK-MG sera=71 were sent from Pisa, Italy by the MG specialist Dr. Ricciardi. Sixty-nine of these sera were selected for the optimisation of the MuSK CBA as one sample was negative by MuSK RIA and one bound non-specifically to HEK cells in CBAs.

There are currently no standardised diagnostic criteria for MG (please see **Section 1.6- Clinical features of myasthenia gravis**). To ensure MG cases were appropriately selected all patients had been seen by MG specialists who provided brief clinical features, and supportive features of (1) neurophysiology (evidence of decrement on repetitive nerve stimulation [decrement of fourth CMAP amplitude greater than 10% of baseline value]) and/or neuromuscular jitter on single-fibre EMG, (2) treatment response to cholinesterase inhibitors, and (3) treatment response to immunotherapy in 184/229 (80%). For further information please see **Section 3.3.4- Clinical features of patients identified by CBAs and Section 3.3.5- Triple-SNMG**.

The diagnosis of MuSK-MG was established on the basis of characteristic clinical features, detection of MuSK-Abs (determined by RIA) and a beneficial response to immunotherapy in 67/69 (97.1%). Neurophysiology was not routinely performed. For further information please see **Section 4.2 Clinical characteristics of MuSK-MG**.

As controls, 4 MuSK-MG, 10 AChR-MG, 10 Aquaporin-4 and 16 N-methyl-D-aspartate receptor-Ab positive serum samples were identified through routine screening by

the Oxford Neuroimmunology diagnostic service. Thirty-five samples from healthy controls (HC) were also provided anonymously with information about age and sex unavailable.

2.4 Radioimmunoassay

MuSK antibody concentrations were determined by radioimmunoassays as previously described (McConville et al., 2004). MuSK-MG sera, purified IgG or IgG subclasses were serially diluted 5-fold in PTX (0.02M PBS, 0.1% Triton X) to a total volume of 50 μ l and incubated with 50 μ l of 125 I labelled MuSK and 5 μ l carrier serum (healthy control serum) over night at 4°C. 50 μ l anti-human IgG was added and incubated at room temperature until precipitation was visible (approximately 60 minutes). After addition of 500 μ l PTX for washing, the samples were centrifuged 5 min at 13000g at room temperature, and then counts per minute (cpm) were measured with a Wallac Wizard counter. AChR or MuSK titres were then calculated using this simplified equation, which corrects the cpm immunoprecipitated into nM.

$$\text{nM} = \frac{(\text{cpm test sample} - \text{cpm negative control}) \times \text{decay factor}}{\text{sera volume}/\mu\text{l} \times \text{specific activity (Ci/mmol)} \times \text{counter efficiency} \times 2.22}$$

2.5 Cell-based assays

HEK-293 cells were detached using trypsin, and centrifuged for 5 minutes at 1100 xg. The cells were re-suspended, counted and plated at a density of 2×10^5 cells/well on Poly-L-Lysine coated 13mm glass coverslips in 6-well cell culture plates. For the MuSK CBA, 3 μ g of MuSK-EGFP cDNA was transfected into HEK cells. For the clustered AChR (clustered-AChR) assay, cells were transiently co-transfected, using polyethylenimine (PEI), with plasmids encoding the four subunits of human adult AChR or foetal AChR and rapsyn-EFGP (with a total of 3 μ g of α : β : δ : ϵ :rapsyn or α : β : δ : γ :rapsyn respectively at 2:1:1:1:1; Leite et al. 2008); (unless otherwise specified the term ‘clustered AChR CBA’ refers to assays

utilising the adult AChR isoform). For the LRP4 CBA, LRP4, covalently linked to the transmembrane and cytosolic domains of CASPR2 (to preserve transmembrane positioning) and LDL receptor-related protein-associated protein 1 pcDNA3.1 were co-transfected with a 5:1 ratio (6µg; Cossins et al. 2012; the plasmids used in these assays have previously been published in the references cited above). For the Aquaporin-4 (AQP-4) CBA, 3µg of AQP-4 pcDNA3.1(+) were transfected into HEK cells. In each case, the medium was changed 16 hrs post-transfection. A list of the constructs used in this project is presented in **Table 2.3**.

Twenty-four hours later, coverslips were transferred to a 24-well cell culture plate and incubated with human sera diluted 1:20 in assay buffer (DMEM, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1% BSA) for 1 hr at RT. After washing and fixing in 3% formaldehyde, coverslips were washed and incubated with one of the following secondary Abs in assay buffer as appropriate: goat anti-human IgG-Alexa Fluor 568 (Invitrogen, binds both heavy and light chains), goat anti-human IgG Fc(γ) (Thermoscientific); goat anti-human-IgM Alexa Fluor 594 (Invitrogen) or IgG subclass specific anti-human IgG1, IgG2, IgG3 or IgG4 (Sigma). For the IgG subclass and Fc(γ) assays the secondary Abs were not fluorescently labeled, and a third layer of goat anti-mouse Alexa Fluor 568 was used. After final washing the coverslips were mounted in mounting media (DakoCytomation, UK) with 1% DAPI (4',6'-diamidino-2-phenylindole dichloride). Slides were read the following day with an Axion Zeiss inverted fluorescent microscope and all photographs taken under identical conditions with MacProbe v4.3 digital image system.

Construct	Species	Vector	Ascension Number	Reference
AChR- α^a	Human	pcDNA3.1	P02708	Leite et al. 2009
AChR- β	Human	pcDNA3.1	P11230	Leite et al. 2009
AChR- δ	Human	pcDNA3.1	Q07001	Leite et al. 2009
AChR- ϵ	Human	pcDNA3.1	Q04844	Leite et al. 2009
AChR- γ	Human	pcDNA3.1	P07510	Leite et al. 2009
Rapsyn	Human	pEGFP-N1	Q13702	Leite et al. 2009
MuSK	Human	pEGFP-N1	O15146	Leite et al. 2009 McConville et al. 2004
LRP4-Caspr2	Human	pcDNA3.1	O75096	Cossins et al. 2012
LRPAP	Human	pcDNA3.1	P30533	Cossins et al. 2012
DOK7	Human	pBABE-IRES-GFP	Q18PE1	Beeson et al. 2006
Agrin ^b	Rat	pcDNA3.1	P25304	Ferns et al. 1993
Aquaporin-4 ^c	Human	pcDNA3.1 ⁺	P55087	Waters et al. 2012

Table 2.3. List and details of constructs used. ^a P3A isoform; ^b c-terminal fragment containing all three splicing sites (amino acids: 1145-1940); ^c M23 isoform.

2.6 Cell-based assay scoring

Scoring of clustered-AChR, LRP4 and MuSK CBAs were performed by two blinded observers as previously described (Leite et al. 2008). Binding of the red fluorescent-labeled secondary antibodies was scored 1-4, based on the degree of cell surface fluorescence and co-localisation with EGFP labeled AChRs or MuSK (**Figure 2.1**). cDNAs used for the LRP4 and AQP4 CBAs did not contain EGFP and only the cell surface staining was scored. Non-specific binding was excluded when sera positive for one antigen were negative for the two other antigens. If sera bound two antigens, negativity on the third antigen was used to exclude non-specific binding to HEK cells. If sera bound three antigens, non-specific binding was assessed using colocalisation and preadsorption studies (see below). Mean end-point titers between anti-human IgG Fc(γ) and anti-human IgG (H+L) were determined by identifying the highest dilution at which the serum gave a score of 1.

2.7 Colocalisation

To confirm specific binding to extracellular MuSK, following incubation with human sera the coverslips were washed and incubated with commercial polyclonal goat anti-MuSK-Ab AF562 (R+D systems) for 1 hour at RT. After washing and fixing in 3% formaldehyde, the coverslips were washed and incubated with goat anti-human IgG-Alexa Fluor 488 (green, Invitrogen) and rabbit anti-goat IgG-Alexa Fluor 568 (red, Invitrogen). Coverslips were then washed and prepared for analysis as previously described.

2.8 Preadsorption

For preadsorption studies the highest positive MG sera (1:10 dilution) was adsorbed sequentially thrice against 9×10^6 live untransfected HEK cells or MuSK transfected HEK cells in solution for 1 hr at RT. The adsorbed sample (equivalent to 1:20 pre-adsorption) was tested by MuSK-CBA to confirm adsorption.

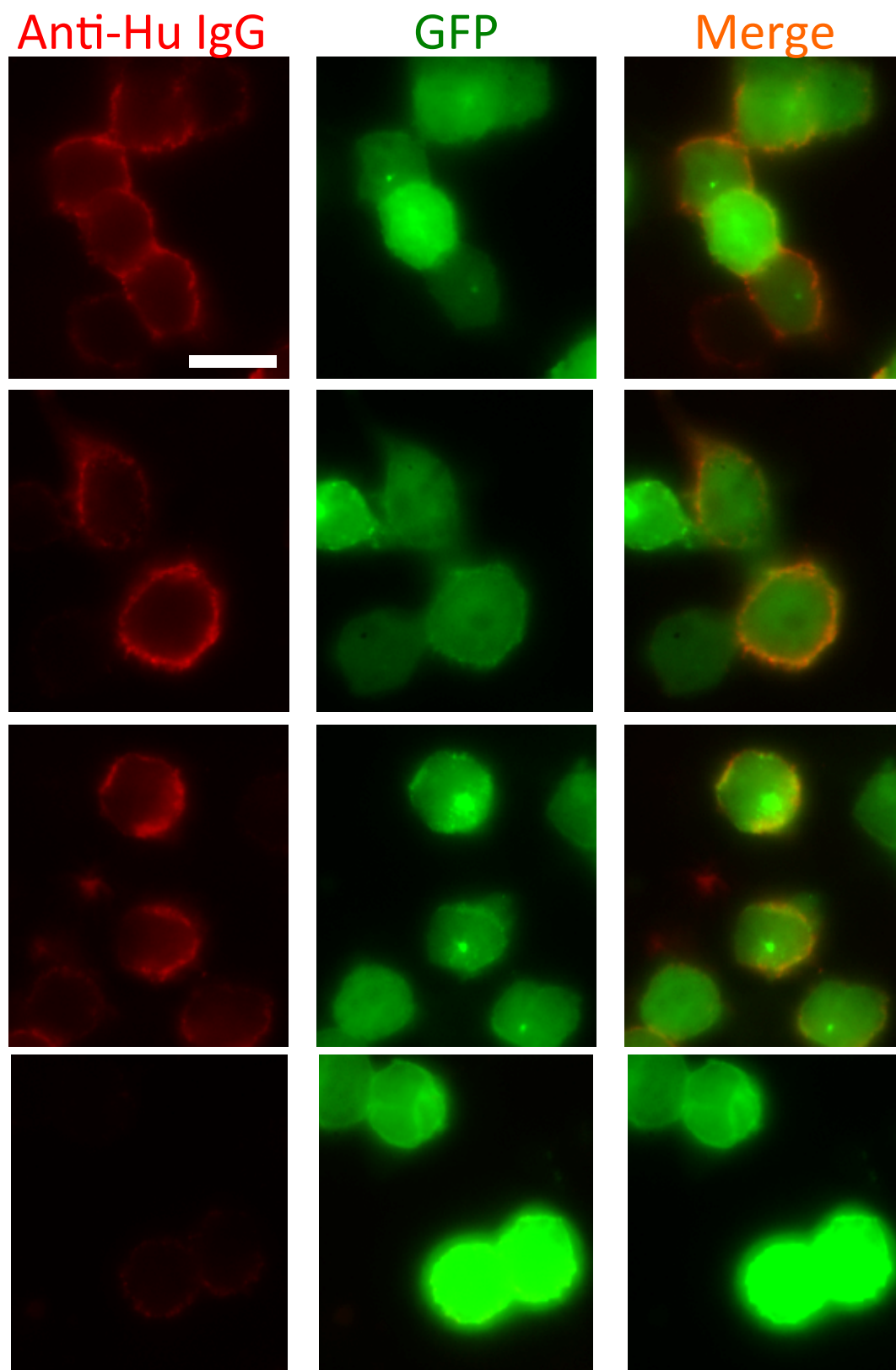


Figure 2.1. Representative images of a clustered-AChR cell-based assay (CBA). *1st row*: CBA score 4, *2nd row*: CBA score 3, *3rd row*: CBA score 2, *4th row*: CBA score 1. Anti-Hu IgG = anti-human IgG; GFP = green fluorescent protein. Magnification 100x, scale bar = 20 μ M

2.9 Agrin production and AChR clustering assays

For production of agrin, a T175 flask of HEK293 cells was transfected with neural agrin (originally donated by the late Dr. Werner Hoch). After 24 hrs the culture media was changed and after 48 hrs the conditioned medium was centrifuged at 1200g for 10min at room temperature, aliquoted, and stored at -20°C. To demonstrate inhibition of AChR clustering by the samples, the sera were heat inactivated at 55°C for 30 minutes, dialyzed, and filter sterilized prior to use. C2C12 myotubes were incubated with patient sera (1:10) for 30 minutes followed by 1:1000 agrin in differentiation medium for 16 hrs. Samples were blinded prior to application. AChR clusters were labeled using Alexa Fluor 594-conjugated α -bungarotoxin (Invitrogen) diluted 1:1000 in differentiation media for 60 minutes at 37°C, 8% CO₂. Myotubes were then washed in differentiation media, fixed in 3% formaldehyde, and washed and stored in PBS at 4°C. Twenty fields containing myotubes were selected with bright field and red fluorescent images were taken using Simple PCI (Digital Pixel) software. Images were analyzed blind for AChR cluster number using a macro with Image J software. For some screening and optimisation experiments, images were analysed with a visual score (1-4) based on the confluence of AChR clusters noted (**Figure 2.2**).

2.10 TE671/CN21 cell-based assays

TE671 and CN21 cells were detached and plated onto 6-well plates containing PLL coated coverslips using the CBA protocol for HEK-293 cells. Coverslips were transferred to a 24-well cell culture plate and incubated with human sera diluted 1:10 in assay buffer (DMEM, 20 mM HEPES, 1% BSA) for 1 hr at RT. After washing and fixing in 3% formaldehyde, the coverslips were washed and incubated with goat anti-human IgG-Alexa Fluor 568 (Invitrogen) in assay buffer. After the final wash coverslips were mounted in mounting media with 1% DAPI.

2.11 DOK7 overexpression in C2C12

For DOK7 overexpressing myoblasts, C2C12 were transfected with DOK-7 using the Phoenix Eco cell line, a retroviral packaging cell line derived from HEK293T cells. Phoenix cells were transfected with pBABE-IRES-EGFP-DOK7 using PEI. After 24 hrs, the growth medium (HEK) was changed. After a further 48 hours the supernatant containing replication incompetent retrovirus with the DOK-7 plasmid was collected, aliquoted, and stored at -80°C. C2C12 cells, 6×10^4 were seeded into a T25 flask. Twenty-four hours later 1mL of retrovirus supernatant with 1mL of polybrene (to enhance efficiency of infection) was added. Twenty-four hour later the cells were passaged into a T175, and after a further 24 hours puromycin was added to the growth medium to select for infected cells. Successful transfection was confirmed by visualising EGFP expression visually using an Olympus IX71 fluorescence microscope.

2.12 Immunoprecipitation from TE671/CN21 cells

TE671 or CN21 cells were grown to confluence in T175cm² flasks as previously described. To avoid cleavage of cell-surface proteins, 1mM EDTA (UltraPure Life Technologies) was substituted for trypsin for the detachment of adherent cells (Huang et al. 2010). Detached cells were then centrifuged (1200g), re-suspended and rotated with patient or healthy control serum (1:10) in DMEM supplemented with HEPES for 60 min at RT. Cells were then centrifuged (1200g), washed three times, and resuspended in ice-cold lysis buffer buffer (10mM Tris-HCl, pH 7.4. 1mM EDTA, 100mM NaCl, 1% Triton X-100, with 1:100 protease inhibitor cocktail). Cell lysates were centrifuged at 13,000g for 15 minutes at 4°C and resultant supernatants incubated with protein-G dynabeads[®] (50µl) for 30 minutes at RT.

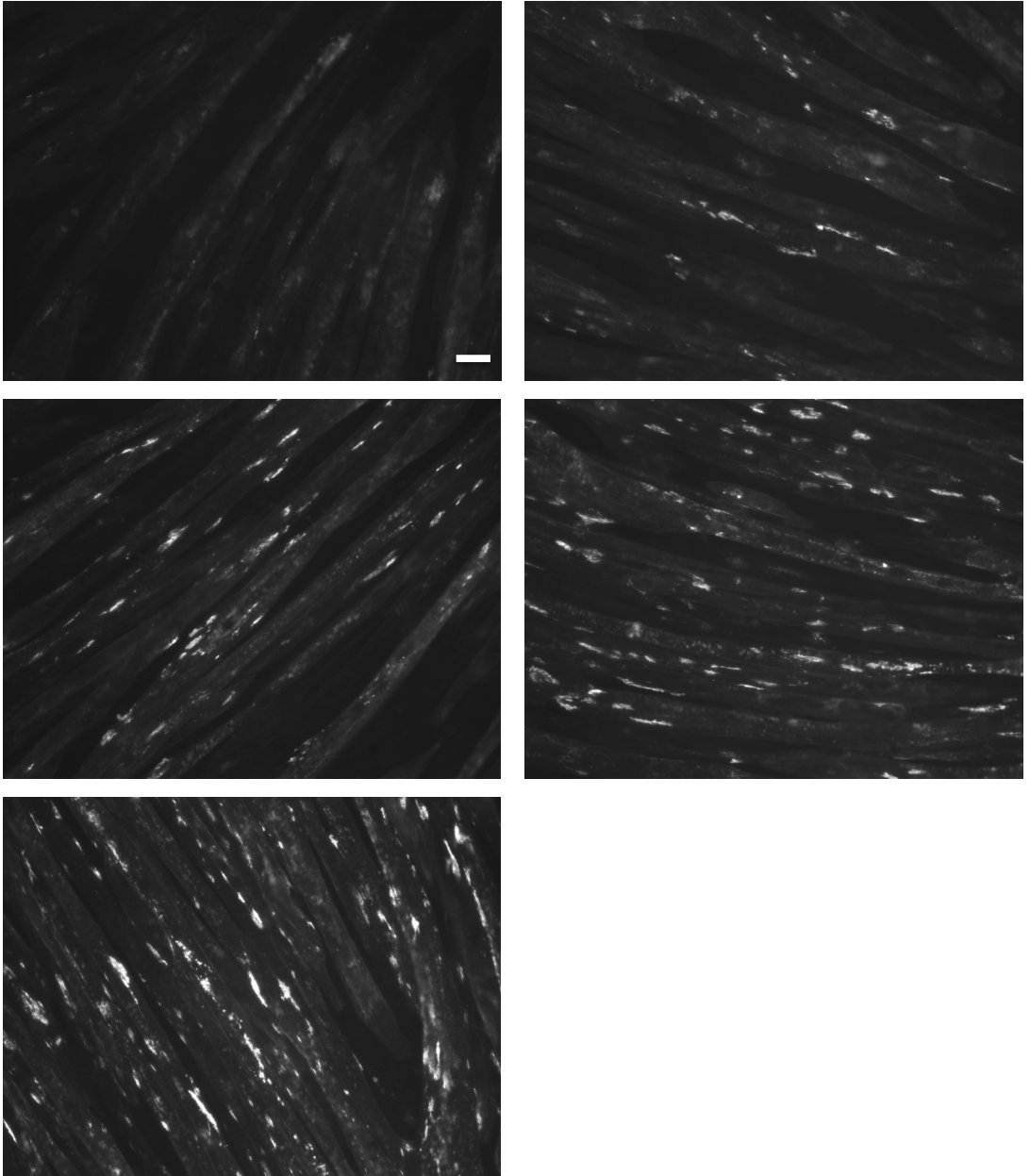


Figure 2.2. Representative images for visual scoring of AChR clusters. *Upper left:* 0/4, *upper right:* 1/4, *middle left:* 2/4, *middle right:* 3/4, *lower left:* 4/4. Magnification 10x, scale bar = 50 μ m

2.13 In-gel trypsin digestion

Protein samples were mixed with reducing SDS-PAGE sample buffer, heat denatured (95°C for 5 min) and resolved on a Tris-Acetate 3-8% gel (150V, 90 min). Following Coomassie blue staining each lane was excised to generate 5 equally sized sections which were cut into 1 – 2 mm³ gel pieces and placed into 1.5 mL sample tubes. Gel pieces were rinsed twice with wash solution for 18hr in total (200µL, 50% methanol, 5% acetic acid). The solutions were removed and gel pieces were dehydrated in acetonitrile (200µL, 5 min). Supernatant were removed and gel pieces were dried in a vacuum centrifuge for 3 min. Disulfide reduction was performed with 10mM DTT (30µL) for 0.5 h, followed by alkylation with 100mM iodoacetamide (30µL) for 0.5 h. Supernatants were removed from the gel samples and dehydration with acetonitrile and evaporation performed as described above. Gel pieces were washed with 100mM ammonium bicarbonate (20µL, 10 min). Supernatants were removed and dehydration performed with acetonitrile and evaporation as above. The gel samples were then rehydrated on ice with freshly prepared trypsin solution (30µL, 20ng/µL sequencing grade trypsin [Promega] in 50mM ammonium bicarbonate). After rehydration excess trypsin solution was removed and 50mM ammonium bicarbonate (10µL) was added to prevent dehydration of gel pieces. Gel samples were digested at 37°C for 18h. The gel pieces were then extracted sequentially with 50mM ammonium bicarbonate (60µL), 50% acetonitrile, 5% formic acid (60µL) and 85% acetonitrile, 5% formic acid (60µL). The combined extracts were evaporated in a vacuum centrifuge and were redissolved in 5% acetonitrile, 0.1% formic acid (20µL) on an ultrasonic bath and transferred into LC-MS sample vials.

In-gel digestion reagents	Preparation method
Wash solution	50% methanol 5% acetic acid in MilliQ-H ₂ O
Ammonium bicarbonate 100mM	0.79g NH ₄ HCO ₃ /100ml MilliQ-H ₂ O
DTT 1M	1.54g dithiothreitol/10ml MilliQ-H ₂ O
DTT 10mM	1.0ml 100mM NH ₄ HCO ₃ 10µl of DTT 1M
50mM iodoacetamide	0.0092g iodoacetamide/1ml MilliQ-H ₂ O
Extraction buffer 1	50% acetonitrile 5% formic acid in MilliQ-H ₂ O
Extraction buffer 2	85% acetonitrile 5% formic acid in MilliQ-H ₂ O
Buffer A	98% MilliQ-H ₂ O 5% formic acid in MilliQ-H ₂ O

Table 2.4 summarises the reagents and composition of solutions used for in-gel digestion.

2.14 LC-MS/MS analysis

Dr. Holger Kramer performed the analysis of in-gel digested protein material and the following is included for information. High performance liquid chromatography (HPLC) was performed using an Ultimate 3000 nano-HPLC system (Dionex, Sunnyvale, CA, USA) comprising a WPS-3000 micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micro-pump, and an SRD-3600 solvent rack controlled by Hystar (Bruker Daltonics, Billerica, MA, USA) and DCMS link 2.0 software. Samples were concentrated on a trapping column Dionex (Sunnyvale, CA, USA), 300 μm i.d., 0.1 cm) at a flow rate of 20 $\mu\text{L}/\text{min}$. For the separation with a C18 Pepmap column (75 μm i.d., 15 cm, Dionex), a flow rate of 250 nL/min was used as generated by a cap-flow splitter cartridge (1/1000). Peptides were eluted by the application of a 30 min multi-step gradient using solvents A (98% H_2O , 2% acetonitrile, 0.1% formic acid) and B (80% acetonitrile, 20% water, 0.1% formic acid):

The liquid chromatography was interfaced directly with a 3D high capacity ion trap mass spectrometer (amaZon; Bruker Daltonics) utilising 10 μm i.d. distal coated SilicaTips (New Objective, Woburn, MA, USA) and nano-ESI mode. SPS parameter settings on the ion trap were tuned for a target mass of 850 m/z , compound stability 100% and a smart ICC target of 250,000. MS/MS analysis was initiated on a contact closure signal triggered by HyStar software (version 3.2). Up to five precursor ions were selected per cycle with active exclusion (0.5 min) in collision-induced dissociation (CID) mode. CID fragmentation was achieved using helium gas and a 30%–200% collision energy sweep with amplitude 1.0 (ions are ejected from the trap as soon as they fragment). The composition and run times for HPLC are summarised in **Table 2.5**.

In-gel digestion reagents	Preparation method
Wash solution	50% methanol 5% acetic acid in MilliQ-H ₂ O
Ammonium bicarbonate 100mM	0.79g NH ₄ HCO ₃ /100ml MilliQ-H ₂ O
DTT 1M	1.54g dithiothreitol/10ml MilliQ-H ₂ O
DTT 10mM	1.0ml 100mM NH ₄ HCO ₃ 10µl of DTT 1M
50mM iodoacetamide	0.0092g iodoacetamide/1ml MilliQ-H ₂ O
Extraction buffer 1	50% acetonitrile 5% formic acid in MilliQ-H ₂ O
Extraction buffer 2	85% acetonitrile 5% formic acid in MilliQ-H ₂ O
Buffer A	98% MilliQ-H ₂ O 5% formic acid in MilliQ-H ₂ O

Table 2.4. Reagents and composition of solutions used for in-gel digestion

Composition (% solvent B)	Run time (min)
2-10	0-3
10-25	3-18
25-50	18-30
50-90	30-30.2

Table 2.5. Composition and run times for high performance liquid chromatography

2.15 Data processing and database searching

Raw LC-MS/MS data were processed and Mascot compatible files were created using DataAnalysis 4.0 software (Bruker Daltonics). Database searches were performed using the Mascot algorithm (version 2.4) and the UniProt_SwissProt database with mammalian taxonomy restriction (v2015.11.26, number of entries 549,832). The following parameters were applied: 2+, 3+ and 4+ ions, peptide mass tolerance 0.3 Da, $^{13}\text{C} = 2$, fragment mass tolerance 0.6 Da, number of missed cleavages: two, instrument type: ESI-TRAP, fixed modifications: Carbamidomethylation (Cys), variable modifications: Oxidation (Met).

2.16 IgG and IgG subclasses purification

Total IgG were purified from plasma of two different MuSK-MG patients using protein G sepharose. 10 ml plasma was filtered using 0.2 μm syringe filters, diluted 1:2 in PBS and incubated overnight with 2.5 ml protein G sepharose at 4°C with gentle shaking. Bead slurry was packed into a column, washed with 50ml PBS, and bound IgG were eluted with 0.1M glycine, pH 2.3. 20 fractions of 1 ml were collected into eppendorf tubes containing 150 μl Tris-HCl pH 8.0 to raise the pH, and analysed for protein content by measuring the OD280 with nanodrop. The 5 fractions with the highest OD280 were pooled and dialysed over night against 200 volumes of Hartmann's solution. For the IgG subclasses purification IgG4 affinity matrix was used in a similar manner as protein G sepharose. 10mls of plasma from the previous MuSK-MG patients was filtered using 0.2 μm syringe filters and diluted 1:2 with PBS, then incubated for 1h at 4°C with 1ml IgG4 affinity matrix and packed into a column. After washing with 3 x 10ml PBS, IgG4 were eluted with 0.1M glycine pH 2.3 in 20 x 1ml fractions into eppendorf tubes containing 150 μl Tris-HCl pH 8 to increase the pH. The 5 fractions with the highest OD280 were pooled and dialysed over night against 200 volumes of Hartmann's solution. The flow-through containing IgG1-3 was loaded a second time onto the IgG4 affinity matrix to ensure complete depletion of IgG4, and the flow-through was

collected and incubated overnight with 2.5ml protein G sepharose and eluted as described for IgG purification.

2.17 MuSK phosphorylation assay

C2C12 cells were seeded at 1.5×10^5 per well in 6-well plates and differentiated into myotubes. Cells were starved for 3 hours with DMEM/1%PSA to reduce basal phosphorylation levels. Myotubes were then incubated with agrin (saturating dilution- 1:100 or non-saturating dilution- 1:800), NSC87877 100 μ m (Tocris®) and/or purified IgG/IgG subclasses (MuSK-Ab- 0.5nM) for 40 minutes at 37°C.

After 40 minutes incubation, myotubes were washed three times in DMEM and resuspended in ice-cold lysis buffer (10mM Tris-HCl, pH 7.4. 1mM EDTA, 100mM NaCl, 1% Triton X-100, with 1:100 protease inhibitor cocktail, and 1:100 phosphatase inhibitor cocktail). Cell lysates were centrifuged at 12000g for 15 min at 4°C. For immunoprecipitation of MuSK from the whole cell lysate, anti-MuSK polyclonal antibody 1:100 (AF562, R&D) was added for 1 hr followed by incubation with 50 μ l Dynabeads® protein G for 30 min at RT. For cell surface immunoprecipitation, MuSK bound to purified IgG or IgG subclass fractions was incubated with 50 μ l Dynabeads® protein G (Invitrogen) for 30 min at RT. The dynabeads were washed 5 times with 400 μ l of lysis buffer, resuspended in 50 μ l of LDS sample buffer containing NuPage reducing agent, and heated for 5 minutes at 95°C. The precipitate and See-Blue® Pre-Stained Standard were subjected to SDS-PAGE using NuPage Bis-Tris mini gels and transferred to nitrocellulose membrane. MuSK was detected with 1:100 anti-MuSK polyclonal antibody (AF562, R&D) and phosphotyrosine with 1:500 anti-phosphotyrosine antibody (clone 4G10®, Millipore). The primary antibodies were each diluted in PBS supplemented with 0.1% Tween and 5% Bovine Serum Albumin (Fisher-Scientific). The primary antibodies were detected using species specific HRP-conjugated secondary antibodies and enhanced chemiluminescence (GE

Healthcare Life Sciences). Densitometry of bands was analysed using ImageJ software and the level of MuSK phosphorylation normalised to levels of immunoprecipitated MuSK, and in some experiments to the levels of agrin-induced phosphorylation.

2.18 AChR cluster assays

C2C12 myoblasts were seeded at a density of 1×10^5 into 24-well plates (Corning) and differentiated as described above. For experiments involving MuSK-MG plasma, samples were heat inactivated at 55°C for 30 minutes, dialyzed, and filter sterilised prior to use.

For agrin-dependent AChR clustering C2C12 myotubes were incubated with patient sera (1:10, 1:30 or 1:90) for 30 minutes followed by 1:100 agrin with or without 100uM of NSC-87877 in differentiation medium for 16 hrs.

For experiments involving agrin-dependent pre-formed clusters agrin 1:1000 was incubated with myotubes for 12 hours, followed by incubation of MuSK-MG plasmas with or without 100uM of NSC-87877 for 6 hours.

For agrin-independent pre-formed clusters DOK7 myotubes were incubated with MuSK-MG plasmas with or without 100µM of NSC-87877 for 13 hours.

For recovery of AChR cluster experiments, myotubes were incubated with agrin 1:1000 for 8 hours followed by application of MuSK-MG plasma for 6 hours, and finally re-application of agrin 1:1000 with or without SHP2 inhibitor for 8 hours. Samples were blinded prior to application. AChR clusters were labeled using Alexa Fluor 594-conjugated α -bungaratoxin (Invitrogen) diluted 1:1000 in differentiation media for 60 minutes at 37°C, 8% CO₂. Myotubes were then washed in differentiation media, fixed in 3% formaldehyde, and washed and stored in PBS at 4°C. Twenty fields containing myotubes were selected with bright field, and red fluorescent images were captured using an Olympus IX71 fluorescence microscope and Simple PCI (Digital Pixel) software. Images were analysed blind for AChR cluster number using a macro with Image J software.

2.19 Statistics

Data were analysed with GraphPad Prism Version 6 and MedCalc 16.4.3. The determination of parametric or non-parametric distribution was based on assessments of normality using the D'Agostino and Pearson omnibus and Shapiro-Wilk test. Comparisons of serial titrations were performed using the Wilcoxon matched-pairs signed rank test. Correlation coefficients were determined using Pearson's and Spearman's tests for parametric and non-parametric data sets respectively. Due to the relatively small sample sizes involved in this project categorical clinical data were analysed using two-tailed Fisher's exact tests. To analyse differences in ordinal or continuous non-parametric data between two groups (e.g. age), Mann-Whitney U tests were performed. To determine the statistical significance of deviation away from an expected distribution (e.g. season of disease onset) the binomial test was used. AChR clustering data derived from at least 13 MuSK-MG sera and at least 3 MuSK phosphorylation experiments were pooled and analysed with one-way ANOVA with Holm-Sidak/Sidak's multiple comparison tests or Kruskal-Wallis tests with Dunn's multiple comparison test. For comparisons involving two independent variables (e.g. SHP2 inhibition and serum dilution) analysis with 2-way ANOVA with the Sidak multiple comparison test were performed. Unless otherwise specified all error bars represent the standard error of the mean.

Chapter 3: Candidate and novel antigen detection in seronegative myasthenia gravis

3.1 Introduction

Seronegative myasthenia gravis (SNMG) refers to MG patients without a detectable MG autoantibody. In some cases this can result in diagnostic difficulty, especially in ocular MG where neurophysiology is less sensitive by virtue of the limited muscles available for sampling. Failure to detect an antibody could be due to sub-optimal assay sensitivity, the presence of a novel as yet unrecognised antigenic target, or absence of a pathogenic antibody. The latter appears unlikely for the following reasons, i) SNMG is not clinically distinct from antibody-positive MG, ii) SNMG responds to immunotherapy, in particular plasma exchange iii) SNMG can be passively transferred to an animal model by injection of patient IgG, and iv) cases of maternal to foetal transfer of SNMG have been reported (Evoli et al. 2003; Sanders et al. 2003; McConville et al. 2004; Lavrnic et al. 2005; Romi et al. 2005; Burges et al. 1994; Townsel et al. 2016; Mossman et al. 1986).

AChR-Abs and MuSK-Abs are typically detected using widely available radioimmunoassays (RIAs). These assays involve the incubation of patient sera with radiolabeled antigenic targets e.g. AChR or MuSK, followed by immunoprecipitation of bound immunoglobulins (Igs) using anti-human IgG, and determination of radioactivity counts after subtraction of background levels. Traditionally, MuSK-MG has referred to

patients positive by MuSK-RIA, AChR-MG for patients positive by AChR-RIA, and ‘SNMG’ for patients negative by AChR- and MuSK-RIA. For the purposes of this thesis, this nomenclature is used throughout.

A few specialised centres also use cell-based assays (CBAs). These utilise human embryonic kidney cells which, when transfected with the subunits of AChRs and the scaffolding protein rapsyn, express at high density the receptors on the cell surface allowing for increased sensitivity of AChR-Ab detection in ‘SNMG’ sera. A MuSK CBA is also available (Leite et al. 2008) but this has not been validated for clinical use. In addition to AChR- and MuSK-Abs, LRP4-Abs have been described in variable proportions of SNMG cohorts (Higuchi et al. 2011; Zisimopoulou et al. 2014; Pevzner et al. 2012; Zhang et al. 2012). Other antigenic targets such as Agrin, and Collagen-Q have also been described using ELISAs (Enzyme-Linked Immunosorbent Assay) or CBAs but their relevance is not well established (Cossins et al. 2012; Gasperi et al. 2014; Zhang et al. 2014; Zoltowska Katarzyna et al. 2015).

The first aim of this chapter was to characterise serologically AChR-, MuSK-, and LRP4-Abs in a large international cohort of MG sera using RIAs and CBAs. The second aim was to assess sera negative by these assays for novel antigenic targets.

3.2 Results

3.2.1 Sera

MG sera=250 were collected from archived and new cohorts sent by MG specialists. In 2001 after the discovery of MuSK-Abs in MG by the Oxford neuroimmunology group (Hoch et al. 2001), a number of MG sera were sent for the detection of MuSK-Abs (Hoch et al. 2001). Archived sera (n=130) previously negative by AChR and MuSK RIAs were collected (Germany=10, Turkey=16, Japan=8, South Korea=13, Philippines=12, UK=71). In addition new cohorts of SNMG patients (n=120) were sent by MG specialists, though

some included AChR-MG or MuSK-MG sera for control purposes (Norway=56, Italy (Rome)=25, and South Africa=39). In addition the samples from South Africa were from patients with generalised MG and more prominent bulbar symptomatology who had not necessarily been tested by RIA.

As controls, MuSK-MG=69 (Pisa, Italy) in addition to 4 MuSK-MG (5.2-20.7 nM), 10 AChR-MG (5.7-11.9 nM), 10 Aquaporin-4-Ab (AQP4-Ab) and 16 N-methyl-D-aspartate receptor-Ab (NMDAR-Ab) identified through routine screening as part of the Oxford Neuroimmunology diagnostic service were used. Thirty-five samples from healthy controls (HC) were also provided anonymously with information about age and sex unavailable.

3.2.2 AChR and MuSK RIAs

As some of the cohorts sent contained known AChR- and MuSK-Ab patients (for control purposes), RIAs were repeated. AChR-Abs (0.75-19.0 nM) were positive in 29/250 (11%) and MuSK-Abs (0.37-1.02nM) were detected in 5/250 (2%) MG sera (**Figure 3.1**).

3.2.3 Clustered AChR CBA

To increase sensitivity of AChR-Ab detection and demonstrate that the antibodies were binding to extracellular epitopes, clustered AChR CBAs with the adult isoform (α :2, β :1, δ :1, ϵ :1 and rapsyn) were performed. Clustered AChR-Abs were detected in 36/250 (14%) of MG sera. Fifteen sera positive by RIA were negative by this CBA. These sera may have been positive due to a) binding of cytosolic epitopes available only in the solution phase of the RIA, b) non-specific binding to the bungarotoxin or other proteins caught within the immunoprecipitate, or c) binding to epitopes of the foetal AChR(γ) (α :2, β :1, σ :1, γ :1 and rapsyn) not present in the routine clustered AChR CBA. To investigate further the 15 sera were re-tested for AChR-Abs using 'hot/cold' radioimmunoassays. A mixture of TE671 and CN21 cell extracts were labelled with iodinated bungarotoxin ('hot') with or without prior incubation of non-iodinated bungarotoxin ('cold'). Each sera was incubated in parallel with

hot and cold extracts, immunoprecipitated, washed, and the resultant radioactivity determined from the difference between ‘hot’ and ‘cold’ extracts (**Figure 3.2**). Fourteen out of 15 sera were negative by hot/cold RIA suggesting that the positive results were not specific for bungarotoxin-bound AChR. Only one serum was positive by hot/cold RIA and was found to bind to clustered foetal AChRs. Representative images of sera positive and negative for clustered AChR-Abs are presented in **Figure 3.3**. Overall, of the 250 MG sera, 15 were positive by RIA and CBA, and 23 were positive by clustered AChR CBAs only (RIA AChR-Ab titre 0.18 - 0.34nM).

3.2.4 MuSK CBA

We next performed MuSK CBAs but surprisingly only 54/69 MuSK-MG positive sera bound detectably (sensitivity 78.3%). Moreover, the specificity of the assays was poor with 4/35 (11%) healthy controls and 3/16 NMDAR-Ab sera (19%) demonstrating positive binding (specificity 86.3%). With this assay, MuSK-Abs by CBA were also detected in 19/104 (18%) MG sera but because of poor specificity, these initial results were deemed unreliable (**Figure 3.4**). Further serological testing was postponed to assess and optimise the MuSK CBA.

3.2.5 Assessment of MuSK CBA

Interestingly, even with the apparently “false positive” results, a positive HC serum binding colocalised with MuSK-GFP surface expression (**Figure 3.5**), suggesting binding of the patient immunoglobulins to MuSK. To determine if this was also the case with MG sera, a sample that scored 3-4 on the MuSK CBA was selected for further study. Binding of this serum immunoglobulin colocalised with surface labeling of MuSK using a commercial MuSK-Ab, as expected for an antibody to MuSK (**Figure 3.6**). Moreover, this reactivity was adsorbed by incubation with MuSK-transfected HEK cells (but not with untransfected HEK cells) confirming that the binding was specific for MuSK (**Figure 3.7**).

Several attempted strategies including testing of sera at 1:50 instead of 1:20 and use of goat serum instead of bovine serum albumin to block non-specific binding did not improve assay specificity. In fact the former resulted in loss of assay sensitivity with MuSK-MG sera.

3.3 Subclasses and Isotype

The high proportion and pathogenic potential of MuSK-IgG4 Abs are a recognised feature in MuSK-MG (McConville et al. 2004; Koneczny et al. 2013; Klooster et al. 2012). In addition it became clear that the secondary antibody commonly used in CBAs was not IgG specific and could cross-react with IgM (Waters et al. 2015). Accordingly we used subclass and IgM specific secondary antibodies to study 12 MG sera (of sufficient remaining volume) positive for CBA MuSK-Abs by anti-human IgG (heavy and light chains; H+L), comparing with two MuSK-MG sera (**Figure 3.8**). The MuSK-MG sera were mainly of IgG class with all subclasses variably represented at the high serum concentration (1:20) tested, but IgM was also present in one of the samples. By contrast, 11/12 MG sera positive with anti-IgG (H+L) showed strong evidence of IgM binding to MuSK but no IgG antibodies, and only one showed moderate IgG1 and IgG2 subclass binding. Moreover, the CBA scores for IgM binding to MuSK correlated broadly with the results obtained with anti-human IgG(H+L) ($r=0.5$ $p<0.0001$; Spearman's test). These results suggested that much of the MuSK-CBA reactivity identified with anti-human IgG(H+L) was due to detection of IgM rather than IgG antibodies binding to MuSK.

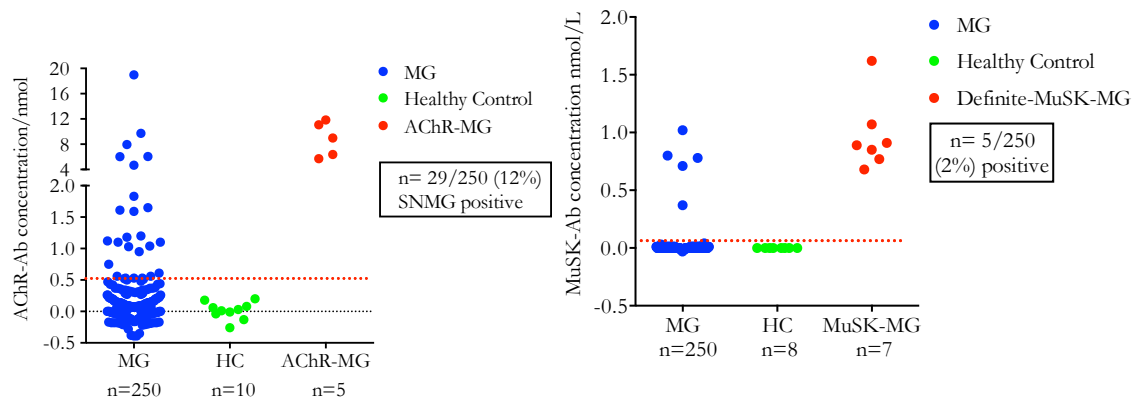


Figure 3.1. AChR and MuSK radioimmunoassays. Scatter plots of results from patients and controls.

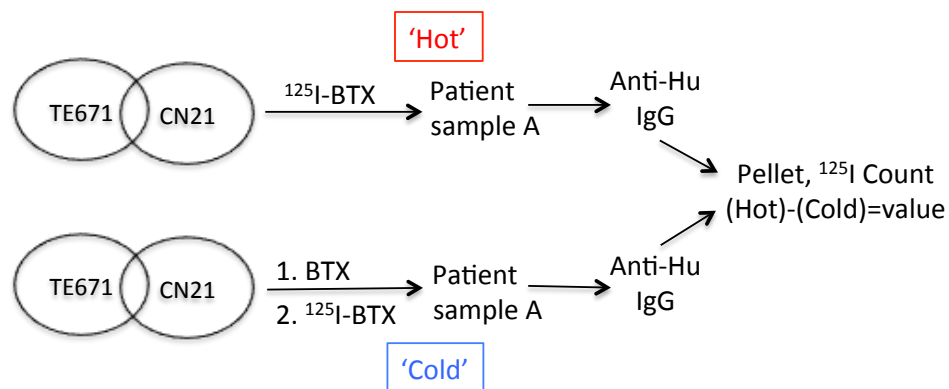


Figure 3.2. Hot/cold radioimmunoassay. Extracts of TE671 (which contain the foetal AChR(γ) isoform) and CN21 (which contain the adult AChR(ϵ) isoform) cells were incubated with 'hot' ^{125}I -bungaratoxin (^{125}I -BTX) or 'cold' followed by 'hot' bungaratoxin. Extracts were then incubated in parallel with a patient serum, and bound IgGs immunoprecipitated using anti-human IgG (anti-Hu IgG). The pellets were then washed and the resultant radioactivity was determined following subtraction of 'cold' counts per minute (cpm) from 'hot' cpm values.

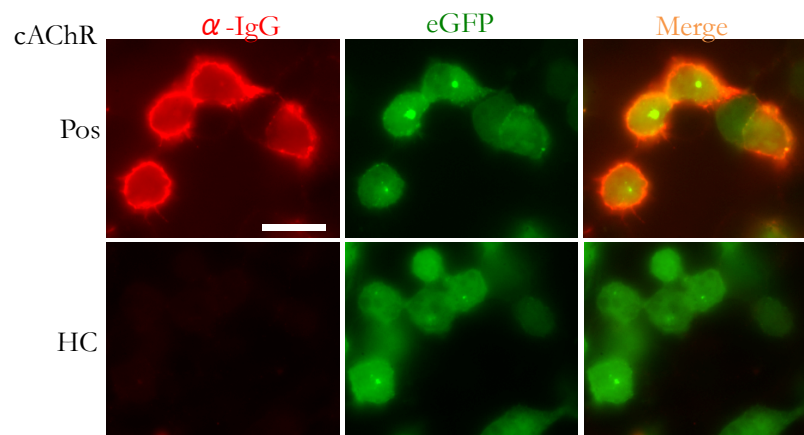


Figure 3.3. Clustered AChR cell-based assay- Anti-human IgG staining (left column, red) and AChR-GFP (middle column, green) colocalise on the merged images (right column, orange). Magnification $\times 100$; scale bar = $20\mu\text{M}$

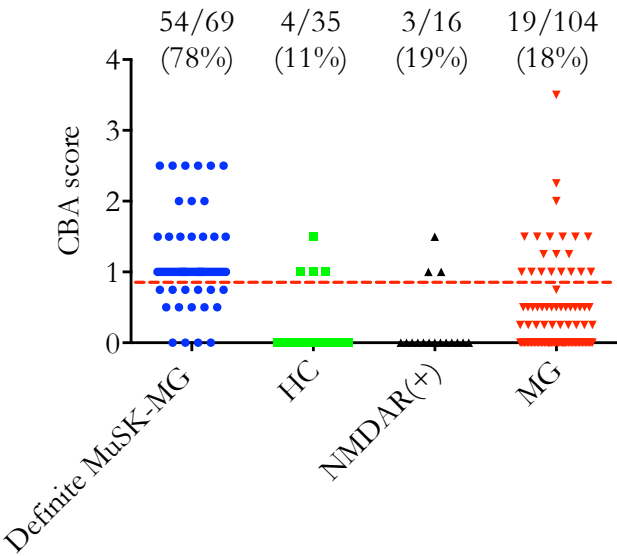


Figure 3.4. CBA with anti-IgG (H+L)- Scatter plots of results from patients and controls

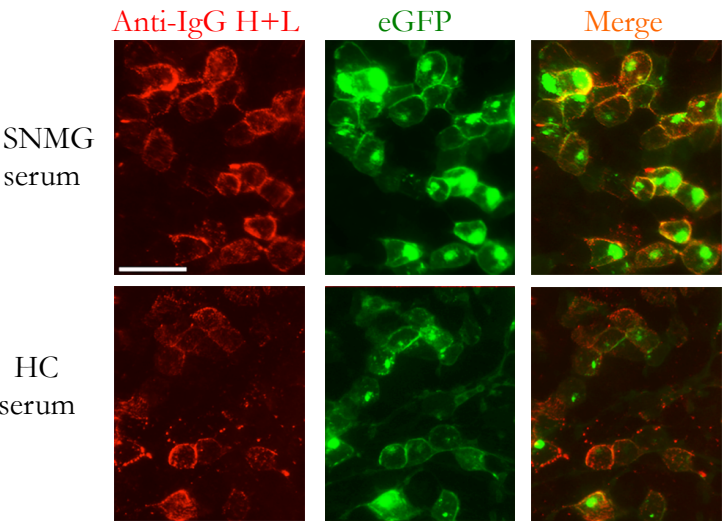


Figure 3.5. Representative CBA images from MG and HC sera. Magnification x40; scale bar= 50μM

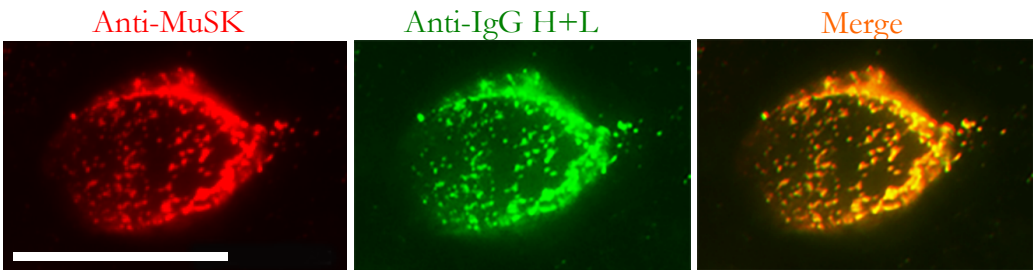


Figure 3.6. Colocalisation of commercial MuSK AF562 antibody (red) with anti-IgG(H+L); (green) in a MG serum. Original magnification 100x (image cropped); scale bar= 20μM

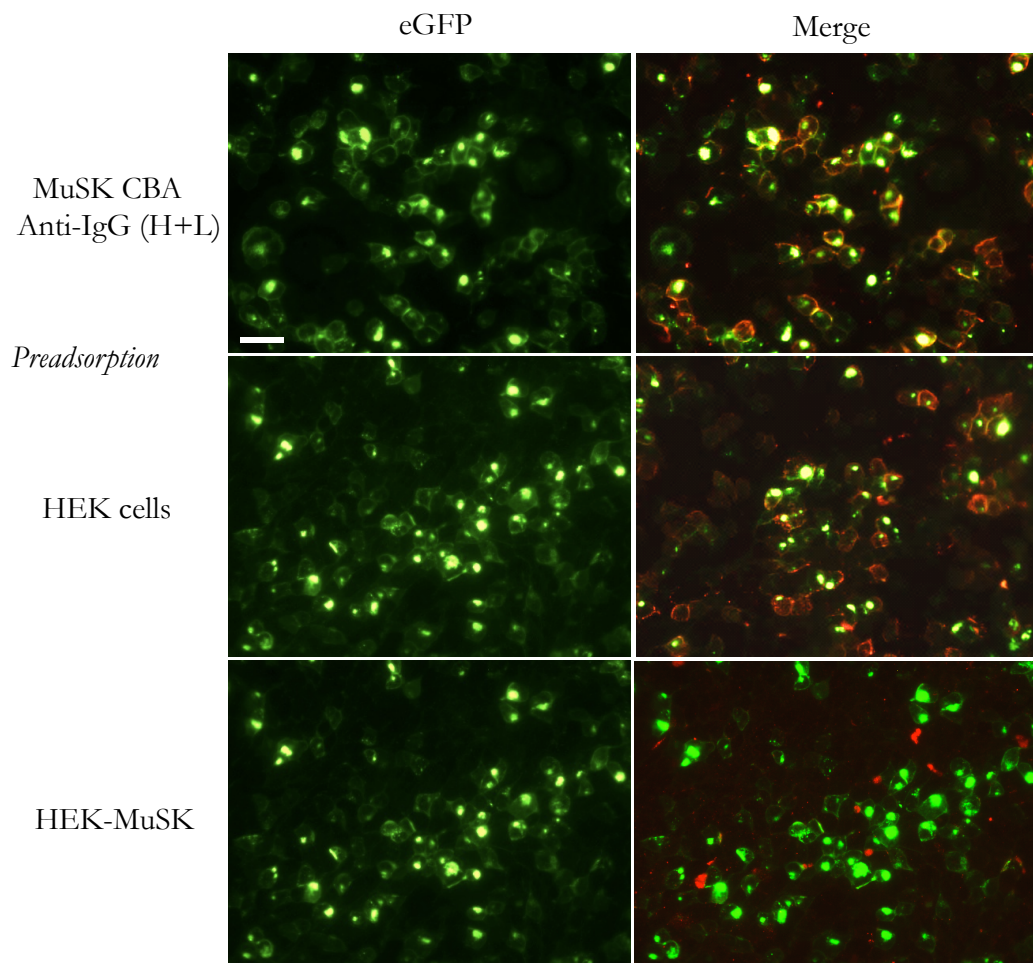


Figure 3.7. Preadsorption- *Upper*. An MG sera that scored 3-4 on the MuSK CBA was preadsorped against HEK cells (*middle*) and HEK cells transfected with MuSK (*lower*). Adsorption only occurred with the latter. Magnification 40x; scale bar= 50μM

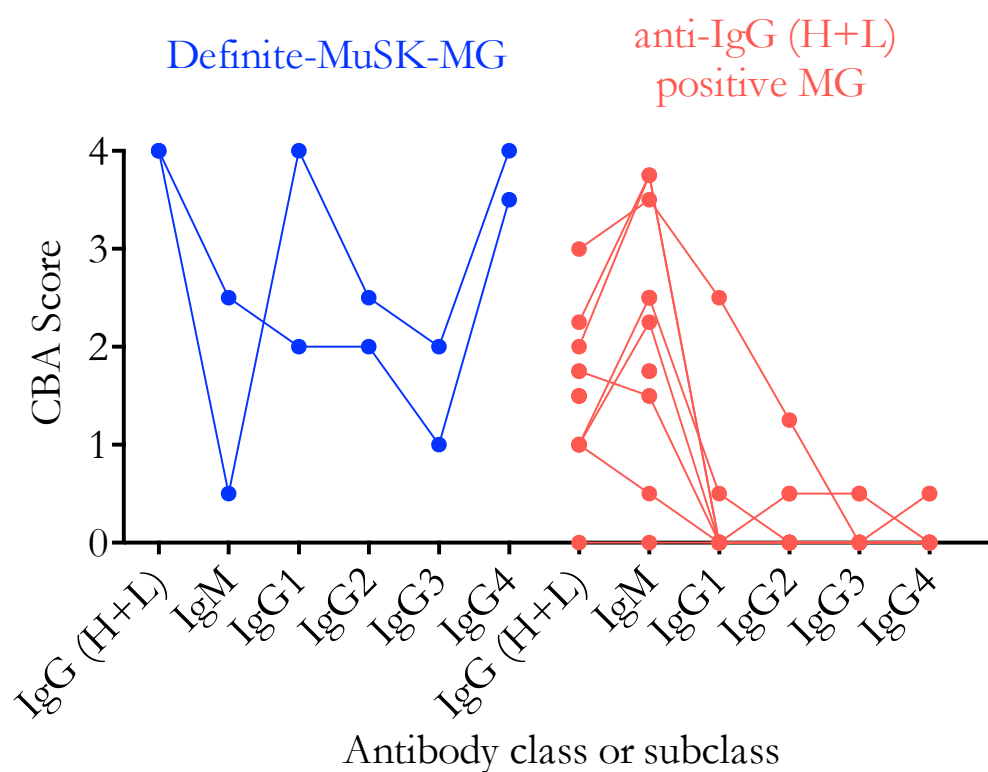


Figure 3.8. IgG subclass and IgM- Antibodies in two MuSK-MG sera were mainly IgG subclasses, particularly IgG1 and IgG4. By contrast, 12 MG sera detected with anti-IgG(H+L) were also detected with anti-IgM secondary antibody but only in one sample with anti-IgG subclass secondary antibodies.

3.3.1 Optimisation of anti-IgG Fc(γ)

The data above confirmed that anti-human IgG(H+L) binds not only to IgG heavy chains, but also cross-reacts with different Igs via the light chains which are not Ig class specific. To improve the CBA for detection of MuSK-IgG Abs, we replaced the anti-human IgG(H+L) secondary antibody with unlabeled anti-IgG Fc(γ) secondary antibody, and detected its binding to human Igs with a tertiary rabbit anti-goat IgG(H+L)-Alexa Fluor 568 antibody.

With this improved IgG-specific method, MuSK-IgG Abs were detected in 68/69 (sensitivity 99% (95% CI 92.2-100)) of MuSK-MG sera. The one negative sample (RIA MuSK-Ab titre 0.91nM) bound non-specifically to HEK cells by CBA and because of limited volume for further examination was designated negative). Moreover, the 35 HC sera, 16 NMDAR-Ab and 10 AQP4-Ab positive sera were negative for MuSK-IgG, demonstrating 100% specificity. A representative CBA image of a MuSK-RIA positive serum is shown in **Figure 3.9**. Four MuSK-MG sera demonstrated higher visual scores and higher end-point dilution titers, with anti-IgG Fc(γ) compared to anti-IgG(H+L); ($p=0.0005$ Wilcoxon matched-pairs signed rank test), likely due to amplification by the tertiary layer (**Figure 3.10**; left). Finally, 15/212 (7%) MG sera from 14 patients, including 6 of the 19 that were positive with anti-IgG (H+L), were positive for IgG specific MuSK-Abs. The frequency distribution data demonstrated fewer borderline results (0.5-0.75) with anti-IgG Fc(γ) as compared to anti-IgG(H+L); ($p<0.0001$; Fisher's test); (**Figure 3.11**). **Figure 3.12** summarises the results of MuSK-Ab testing with goat anti-human IgG (H+L) and IgG Fc(γ).

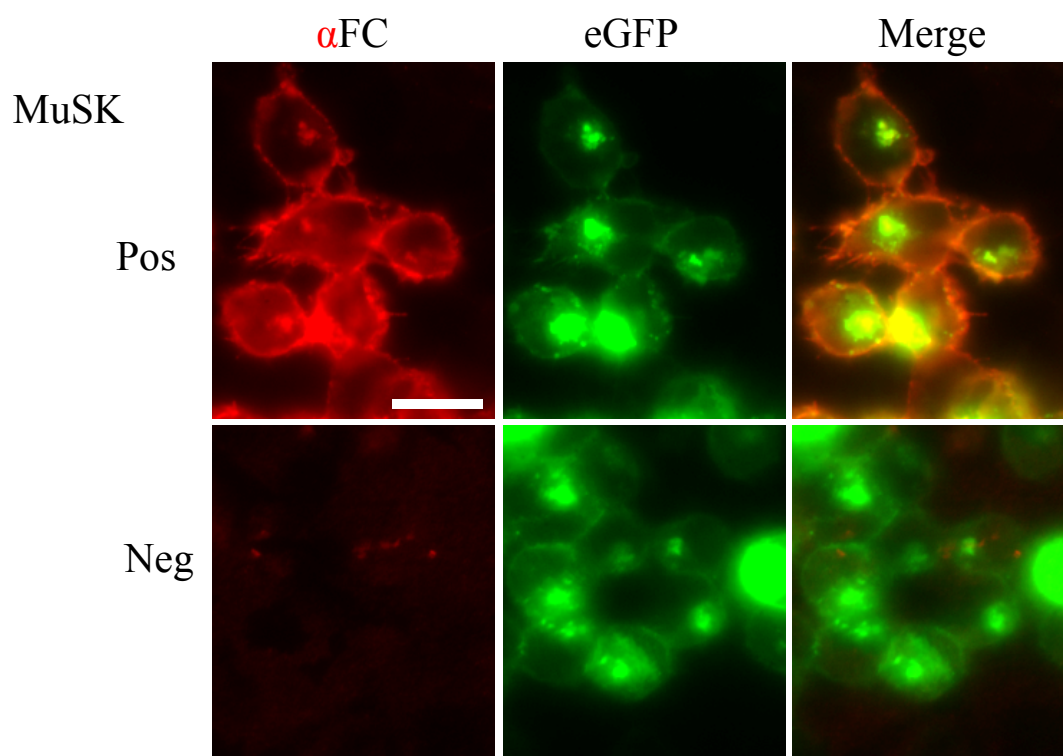


Figure 3.9. Representative images of optimised MuSK cell-based assay. The secondary antibody, anti-human IgG (heavy and light chains) was replaced for anti-human IgG Fc(γ). Representative images of a MuSK-CBA positive MG serum (*upper*) and negative healthy control serum (*lower*) are shown. Magnification 100x; scale bar = 20 μ M

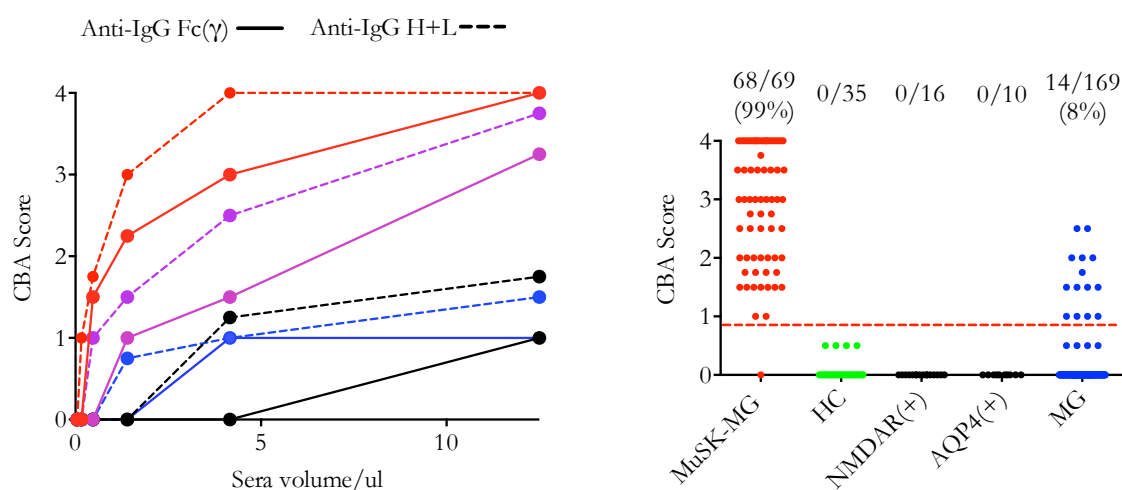


Figure 3.10. *Left:* serial titrations of 4 MuSK-MG sera were compared by MuSK cell-based assays using either anti-human IgG (heavy and light chains) or anti-human IgG Fc(γ). MuSK-MG sera demonstrated higher visual scores and higher end-point dilution titers, with anti-IgG Fc(γ) compared to anti-IgG(H+L); ($p=0.0005$ Wilcoxon matched-pairs signed rank test) *Right:* Scatter plots of patients and controls with a MuSK cell-based assay incorporating anti-human IgG Fc(γ).

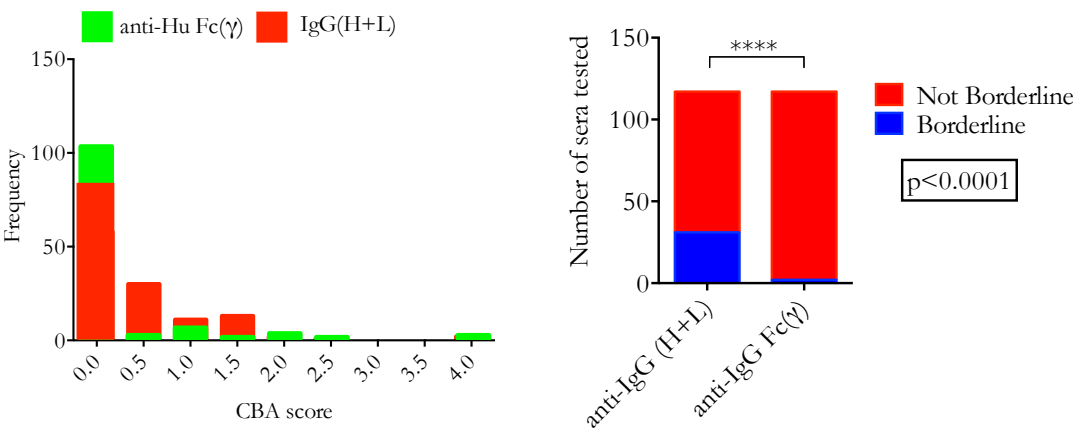


Figure 3.11. *Left:* Frequency distribution of MuSK cell-based assay results (n=117) with anti-human IgG (heavy and light chains) or anti-human IgG Fc(γ). *Right:* Comparison of proportion of borderline cell-based assay results (CBA score: 0.5-0.75) using either anti-human IgG (heavy and light chains) or anti-human IgG Fc(γ); (two-tailed Fisher's exact test $p<0.0001$)

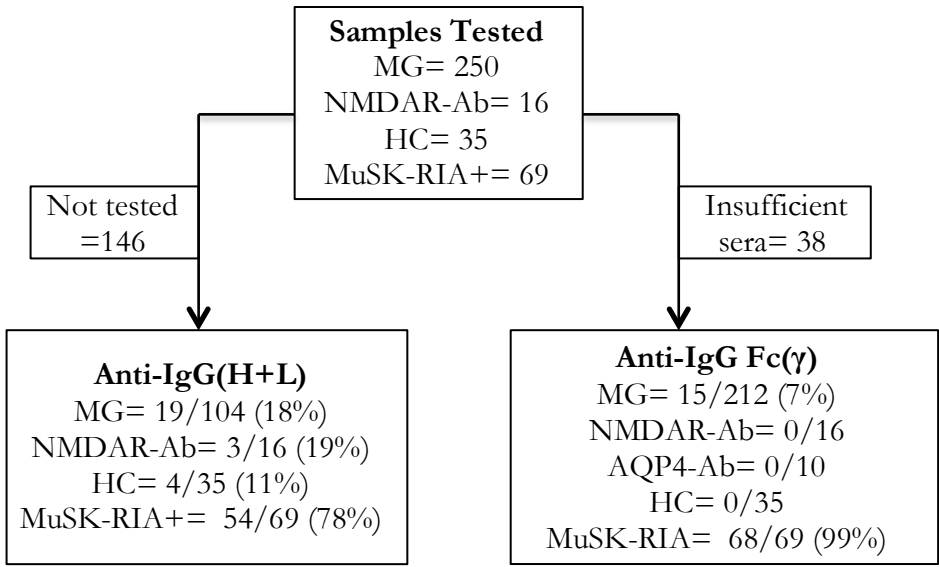


Figure 3.12. Flow chart of results of patients and controls. MG = myasthenia gravis; Ab = antibody; NMDAR = N-methyl-D-aspartate receptor-antibody positive; AQP4 = Aquaporin-4-antibody; HC = healthy control; MuSK-RIA+ = myasthenia gravis with MuSK antibodies positive by radioimmunoassay.

3.3.2 LRP4-, Agrin- and Col-Q-Antibodies

To detect LRP4-Abs in MG sera we used an LRP4 CBA that had previously been optimised (Cossins et al. 2012). Surface expression of LRP4 was confirmed with a commercial LRP4-Ab and with two known LRP-Ab positive sera (**Figure 3.13**). All 7 HCs were negative. LRP4-Abs were detected in only 3/249 MG sera. Of these, one serum was also positive for by AChR-RIA another by MuSK-RIA and MuSK-CBA. Thus only one serum was positive for LRP4-Abs only. Agrin- and Col-Q-Abs were initially screened in HC sera but a high proportion of these were positive (6/10), and further testing of these Abs was not pursued (data not shown).

3.3.3 Summary

Of n=250 MG sera, 15 were RIA-AChR positive and 5 were MuSK-MG. Of the 231 remaining MG sera, Abs were identified by CBAs in 39/231 (17%) sera ((cAChR-Abs; 23/231 (10%), MuSK-IgG Abs; 15/212 (7%), LRP4-Abs; 3/229 (1%, of which 2 were positive by other assays)). The remaining 191 sera were designated triple-SNMG. **Figure 3.14** summarises the results of candidate antigen screening in MG sera.

3.3.4 Clinical features of patients identified by CBAs

Clustered AChR-Abs were detected in 23 patients negative by AChR-RIA (0.18 - 0.34nM); (Table 7). The age of onset of patients followed largely a normal distribution with a median of 42 (6-78) yrs (**Figure 3.15**).

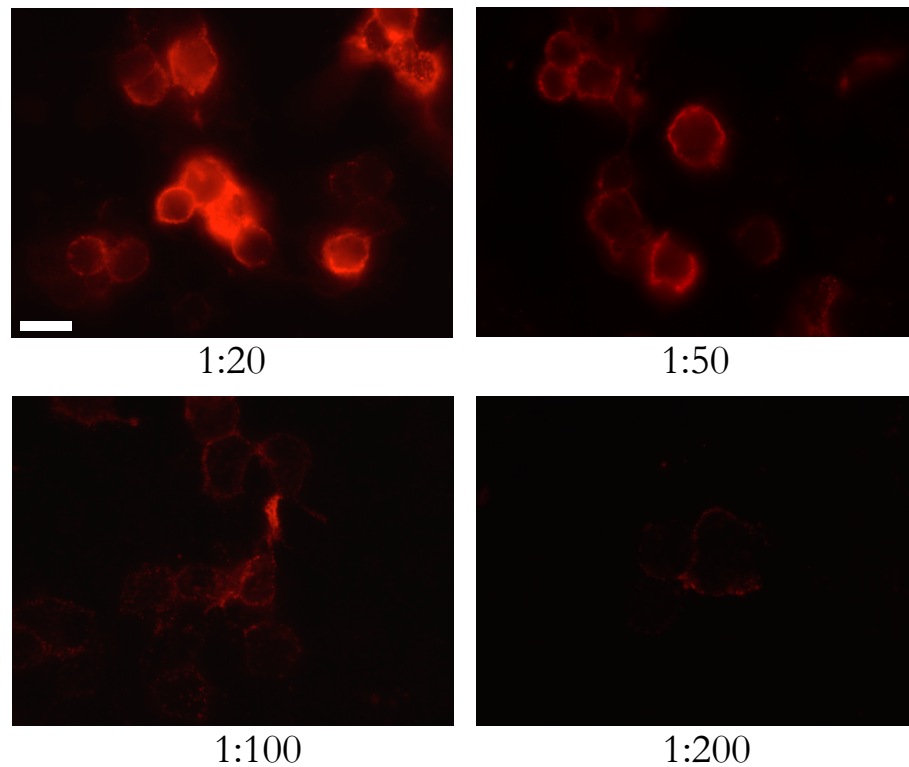


Figure 3.13. LRP4 cell-based assay- Serial dilutions (1:20-1:200) of a LRP4-MG serum were tested by LRP4 cell-based assay. Representative images of the results are shown. The *upper left* image scored 4 whilst the *lower right* image scored 1. Magnification 100x; scale bar = 20 μ M

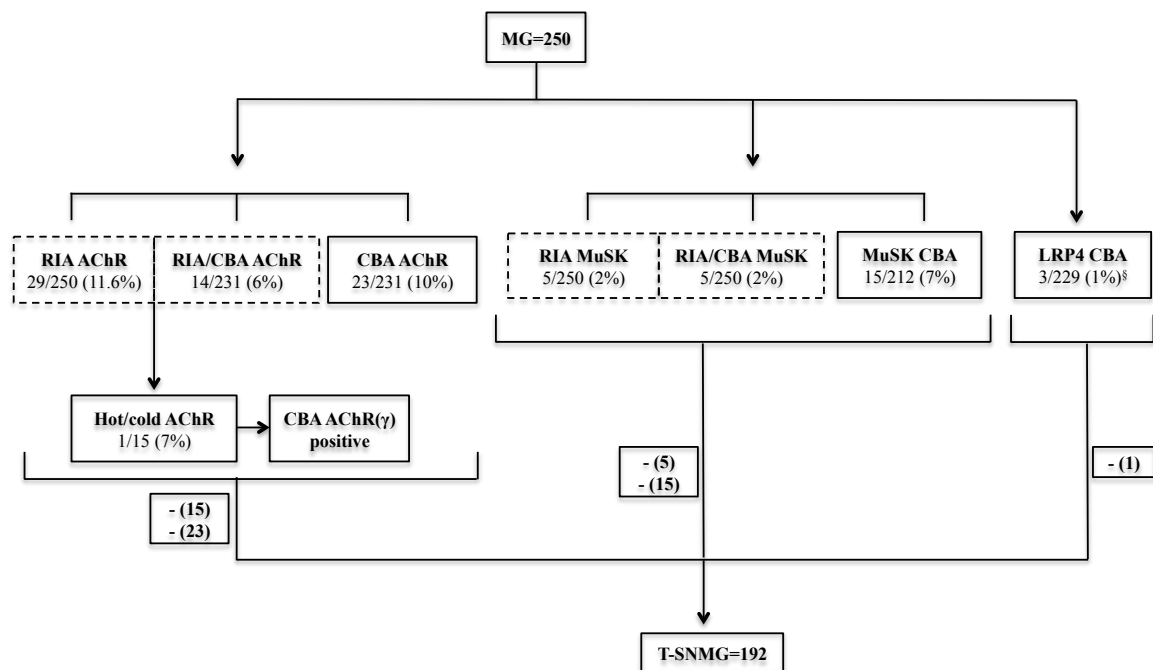


Figure 3.14. Flow chart of serological testing of myasthenia gravis sera. SNMG = seronegative myasthenia gravis; Ab = antibody; cAChR(ϵ) = clustered adult AChR; cAChR(γ) = clustered foetal AChR; RIA = radioimmunoassay; CBA = cell-based assay; triple-SNMG = triple-SNMG; § = one LRP4 CBA positive sample was also positive by MuSK RIA.

Patients were more likely to be female (F:M; 2:1). Ocular confined disease was seen in 7/15 (46.7%) of patients over a median follow-up period of 6 (1-31) yrs. Overall the disease course was relatively mild with MGFA at onset >2 in 2/15 (13.3%), 6/15 (40%) at nadir, and 2/18 (11.1%) at follow-up. Despite this, prominent bulbar/respiratory/axial and limb symptoms were reported by 6/16 (37.5%) and 2/16 (12.5%) patients respectively. Neurophysiology was supportive in 8/14 (57.1%) of cases and a beneficial response to AChEIs was seen in 10/12 (83.3%) patients. Thymectomy was performed in 3/15 (20%) patients and was beneficial in one patient (JG) with outcome data. Thymic histology was atrophic, and in one other patient demonstrated hyperplasia. Patients were treated with therapies including AChEIs only, or combinations with prednisone, azathioprine, or mycophenolate mofetil. Immunotherapy was beneficial in 13/14 (92.9%) of patients; 10/16 (62.5%) achieved a post intervention status (PIS) of minimal manifestations/remission and remission specifically in 3/17 (17.7%) patients. The clinical information of clustered-AChR positive patients is summarised in **Table A.1** in Appendix A.

For further investigation of MuSK-IgG CBA positive sera and clinical comparisons with MuSK-MG patients please see Chapter 4.

LRP4-Abs were detected in 3 patients. One patient of South African origin, IV presented at the age of 19 yrs. with severe oculobulbar symptoms (MGFA 3b). MuSK-Abs were also positive by both RIA and CBA. The MGFA improved to 2b following the introduction of prednisone and azathioprine. MG1130 (Norway) presented at 44 yrs with fatigable ptosis and diplopia. Single fibre EMG of the orbicularis oculi muscles was positive and clustered AChR-Abs were also detected. Only symptomatic treatment with AChEIs was required. Unfortunately no clinical information was available for the final patient RR77 with LRP4-Abs only.

3.3.5 Triple-SNMG

The age at which triple-SNMG patients presented appeared relatively symmetrical with a wide distribution (20-60 yrs), and a median onset age of 40 (1-79) yrs. Patients were more likely to be female (F:M; 2:1) but this was not observed in older age groups (51-60 and 71-80 yrs); (**Figure 3.16**). MGFA at onset was >2 in 19/89 (21.3%), 47/94 (50.0%) at nadir, and 32/147 (21.8%) at follow-up. Ocular MG was present in 19/94 (20.2%) of patients. Ocular symptoms were the most prominent symptom in 58/99 (58.6%), followed by axial/bulbar/respiratory in 39/99 (39.4%), and limb muscles in 24/99 (24.2%) of patients. NCS/EMG was positive in 70/124 (56.5%) and a beneficial response to cholinesterase inhibitors was seen in 87/108 (81.1%).

Immunotherapy with steroids, azathioprine, methotrexate, mycophenolate mofetil, or cyclophosphamide was utilised in 147/191 (77%) of patients. Rescue therapies with intravenous immunoglobulins and plasma exchange were required in 18/135 (13.3%) patients. One patient JR(a) received Rituximab for rheumatoid arthritis, and a subsequent improvement in myasthenic symptoms was noted. Overall, immunotherapy was beneficial in 81/97 (83.5%) of patients. A PIS of minimal manifestations/remission was achieved in 60/123 (48.8%) of patients but remission rates were low- 22/123 (17.9%).

Comparison of female and male patients demonstrated that male patients were older (49 (1-79) v.s. 36 (0-76) yrs; $p=0.0005$; Mann-Whitney test) but interestingly had a less severe disease nadir (MGFA >2 - female 46/66 (69.7%) v.s. male 10/33 (30.3%); $p=0.0003$; Fisher's test). This may have been due to a trend towards a higher proportion of ocular MG in male patients (female 9/66 (13.6%) v.s. male 10/33 (30.3%); $p=0.06$; Fisher's test). Nevertheless MGFA at onset and follow-up, pattern of muscle involvement, NSC/EMG, immunoresponsiveness, and treatment outcomes were similar between male and female patients (**Table 3.1**).

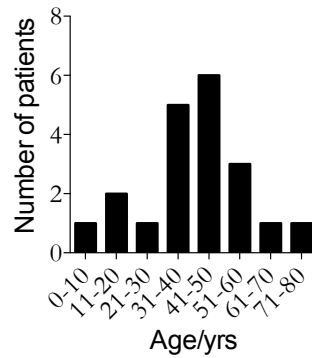


Figure 3.15. Frequency distribution of onset age in 20 clustered-AChR positive patients

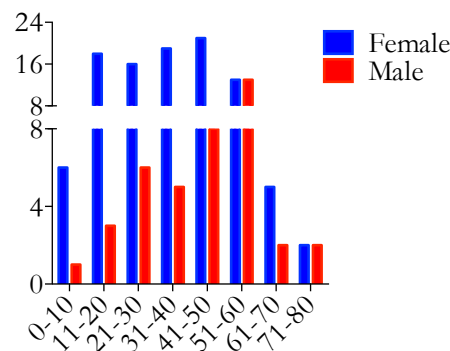


Figure 3.16. Frequency distribution of onset age in patients (male= 40, female= 100) with triple-seronegative myasthenia gravis.

Clinical Parameter	EOMG <40yrs	LOMG ≥50yrs	p-value	Female	Male	p-value
Age	-	-	-	36(0-76) yrs	49(1-79) yrs	<0.01^a
Female	55/69 (79.7)	21/42 (50)	<0.01	-	-	-
MGFA>2						
-onset	7/33 (21.2)	7/24 (29.2)	0.54	15/59 (25.4)	8/34 (23.5)	1.00
-nadir	28/42 (66.7)	11/24 (45.8)	0.12	46/66 (69.7)	10/33 (30.3)	<0.001
-F/U	15/64 (23.4)	12/38 (31.6)	0.49	23/102 (22.5)	5/46 (10.9)	0.11
OMG	8/24 (33.3)	6/24 (0.25)	0.75	9/66 (13.6)	10/33 (30.3)	0.06
Worst symptom(s)						
-ocular	18/34 (52.9)	13/26 (0.50)	1.00	38/64 (59.4)	20/35 (57.1)	0.84
-bulbar	6/34 (17.7)	12/26 (46.2)	0.28	22/64 (34.4)	14/35 (40.0)	0.66
-limb	7/34 (20.6)	5/26 (19.2)	1.00	16/64 (0.25)	8/35 (22.9)	1.00
-resp	2/34 (5.9)	3/26 (11.5)	0.64	7/64 (10.9)	3/35 (8.6)	1.00
-axial	1/34 (2.9)	2/26 (7.7)	0.57	6/64 (9.4)	1/35 (2.9)	0.42
Immuno response	38/42 (90.5)	22/26 (84.6)	0.47	22/64 (34.4)	14/35 (40.0)	0.66
Remission	10/54 (18.5)	4/29 (13.8)	0.76	26/90 (28.8)	8/33 (24.2)	0.66
PIS≥MM	29/54 (53.7)	13/29 (44.8)	0.50	55/90 (61.1)	17/33 (51.5)	0.41

Table 3.1. Comparison between EOMG/LOMG and female/male patients with triple-seronegative myasthenia gravis- EO = early onset; LO = late onset; yrs = years, MGFA = Myasthenia Gravis Foundation America; OMG = ocular myasthenia gravis; resp = respiratory; PIS = post intervention status; MM = minimal manifestations. Unless otherwise specified tests were performed with the Fisher exact test. ^aMann-Whitney test.

Comparison of EOMG patients (age<40 yrs) and LOMG (age≥50 yrs) demonstrated a difference in proportion of female patients as described above ((EOMG 55/69 (79.7%) v.s. 21/42 (50%); $p=0.002$; Fisher's test; **Table 3.1**). Disease course, pattern of muscle involvement, proportion of ocular MG, supportive NSC/EMG, immunoresponse, and treatment outcomes were similar in both groups (data not shown).

3.3.6 Functional effects of triple-SNMG on the agrin/LRP4/MuSK/DOK7 pathway

To determine if triple-SNMG sera affected the agrin/LRP4/MuSK/DOK7 pathway, 14 sera of sufficient (but limited) volume were tested for effects on agrin induced AChR clusters in cultured C2C12 myotubes. As shown in **Figure 3.17**, on visual inspection 3/16 triple-SNMG sera tested appeared to reduce agrin dependent AChR clusters as compared to healthy control sera, but the effects were less marked than with MuSK-RIA positive sera. These results suggest that a minority of triple-SNMG sera may contain antibodies that affect the agrin/LRP4/MuSK/DOK7 pathway and/or AChR density.

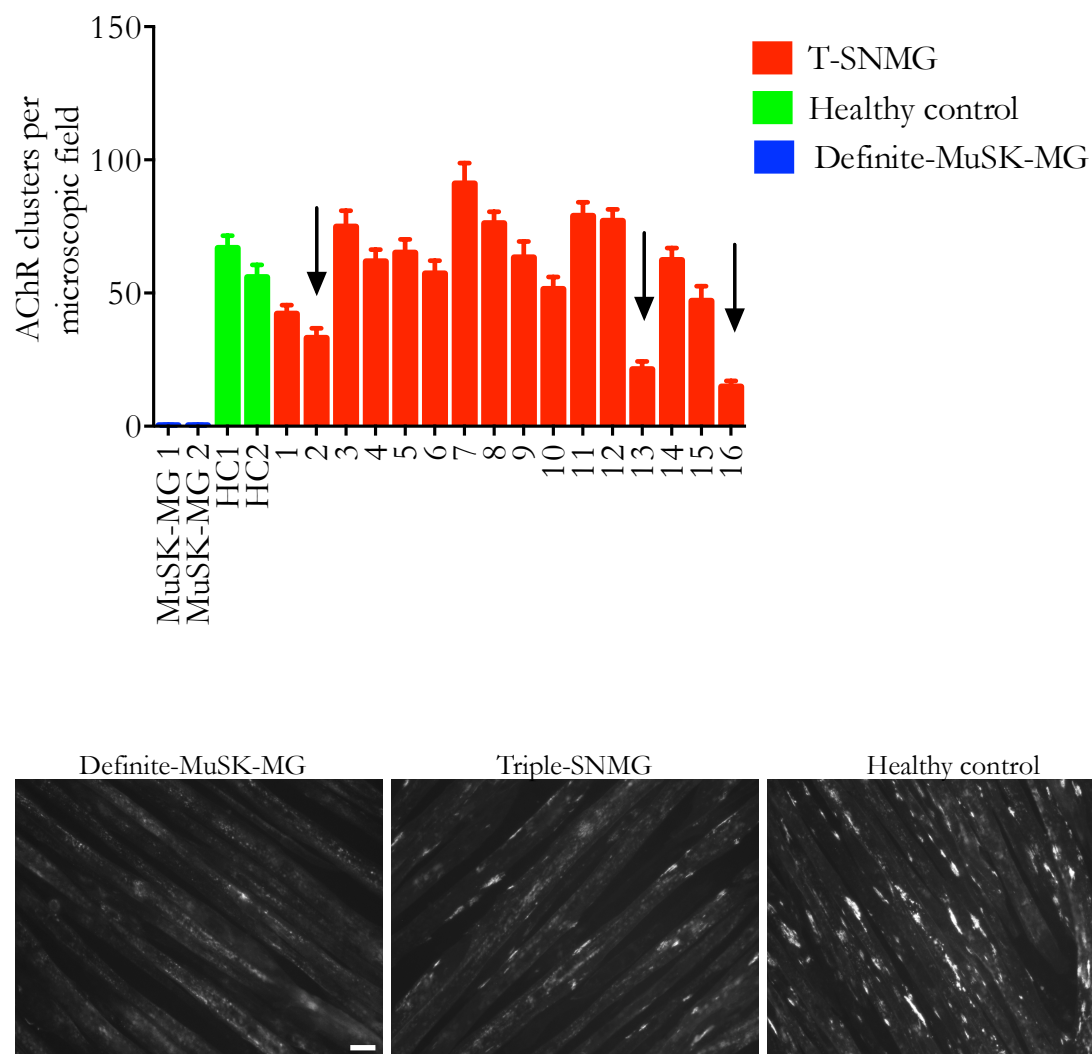


Figure 3.17. *Upper-* Effect of 16 triple-SNMG (T-SNMG; red) sera on agrin dependent AChR cluster formation. Three triple-SNMG sera reduced the number of AChR clusters as compared to 2 healthy controls (green), but less effectively as compared to 2 MuSK-MG sera (red). *Lower:* Representative images of myotubes incubated with agrin and MuSK-MG, triple-SNMG, or healthy control sera. Magnification- 10x; scale bar= 50 μ M

3.3.7 LC-MS/MS of triple-SNMG sera

To determine if triple-SNMG sera contained antibodies against novel antigens the TE671 and CN21 rhabdomyosarcoma cell lines were used. These muscle cell lines express the foetal and adult AChR isoforms respectively, as well as other muscle antigens. Previous experiments using fluorescent activated cell sorting (FACS) have shown that some sera negative for AChR-Abs do bind to these cell lines, and these sera were later shown to contain MuSK-Abs (Blaes et al. 2000). An approach for the successful immunoprecipitation (IP) of MuSK from these cell lines using these sera has also previously described (Scuderi et al. 2002). Moreover, this approach could also be used to identify MuSK in an unbiased approach using liquid chromatography and MALDI (matrix-assisted laser desorption/ionisation) mass spectrometry (Littleton et al. 2009).

To apply this strategy for novel antigen discovery, a cell-based assay was optimised to screen rapidly large numbers of triple-SNMG sera for binding to TE671 and CN21 cells. Live TE671 and CN21 cells were incubated with sera for 1 hr at room temperature, fixed, washed, and then stained for bound IgGs using fluorescent conjugated anti-human IgG. As expected AChR-MG control sera bound more strongly to CN21 cells, which are stably transfected with the adult AChR isoform and have higher levels of AChR surface expression (Beeson et al. 1996). Binding of MuSK-MG control sera appeared to be similar with CN21 and TE671 cells. Representative images of TE671/CN21 surface staining are shown in **Figure 3.18**. However, only 3/10 AChR-MG, 3/4 MuSK-MG and 5/170 (2.9%) triple-SNMG sera bound appreciably to the cell surface of TE671 and/or CN21 cells (**Figure 3.19**). Sera that bound to TE671/CN21, but also bound HEK cells non-specifically were designated negative. Several unsuccessful strategies were attempted to increase binding of positive control sera to TE671/CN21 cells including; higher sera concentrations, increased duration of incubation (1hr vs. 2hrs vs. 3hrs), higher temperature (RT vs. 37°C) or prior incubation with agrin (16 hrs). Despite these efforts only 3 triple-SNMG sera bound to

TE671/CN21 cells. 'FIH22' bound more strongly to CN21 cells whilst 'CW' and 'RR71' bound more strongly to TE671 cells. Two further sera bound appreciably to TE671 cells but were of insufficient volume for further tests.

The IP protocol was then tested with a HC and a MuSK-MG serum. The protocol described by Littleton et al. was modified such that EDTA (Ethylenediaminetetraacetic acid) was used for cell detachment instead of trypsin, so as to avoid cleavage of cell surface proteins (Huang et al. 2010). In addition sera (1:10) were diluted in Dulbeccos's Modified Eagle's Medium (DMEM) supplemented with the buffering agent HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 0.1% bovine serum albumin, as per the protocol for a CBA using HEK cells. TE671 cells derived from 6x T175 flasks were used and incubated with sera for 1 hr. The cells were washed three times, lysed, and incubated with protein G beads for 30 minutes at RT. The IgGs and any proteins bound to them were subsequently eluted, separated by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and probed for MuSK by Western blot. **Figure 3.20** (left) summarises the experimental steps of the IP procedure.

Figure 3.20 (right) demonstrates a representative Western blot of MuSK successfully detected using this method.

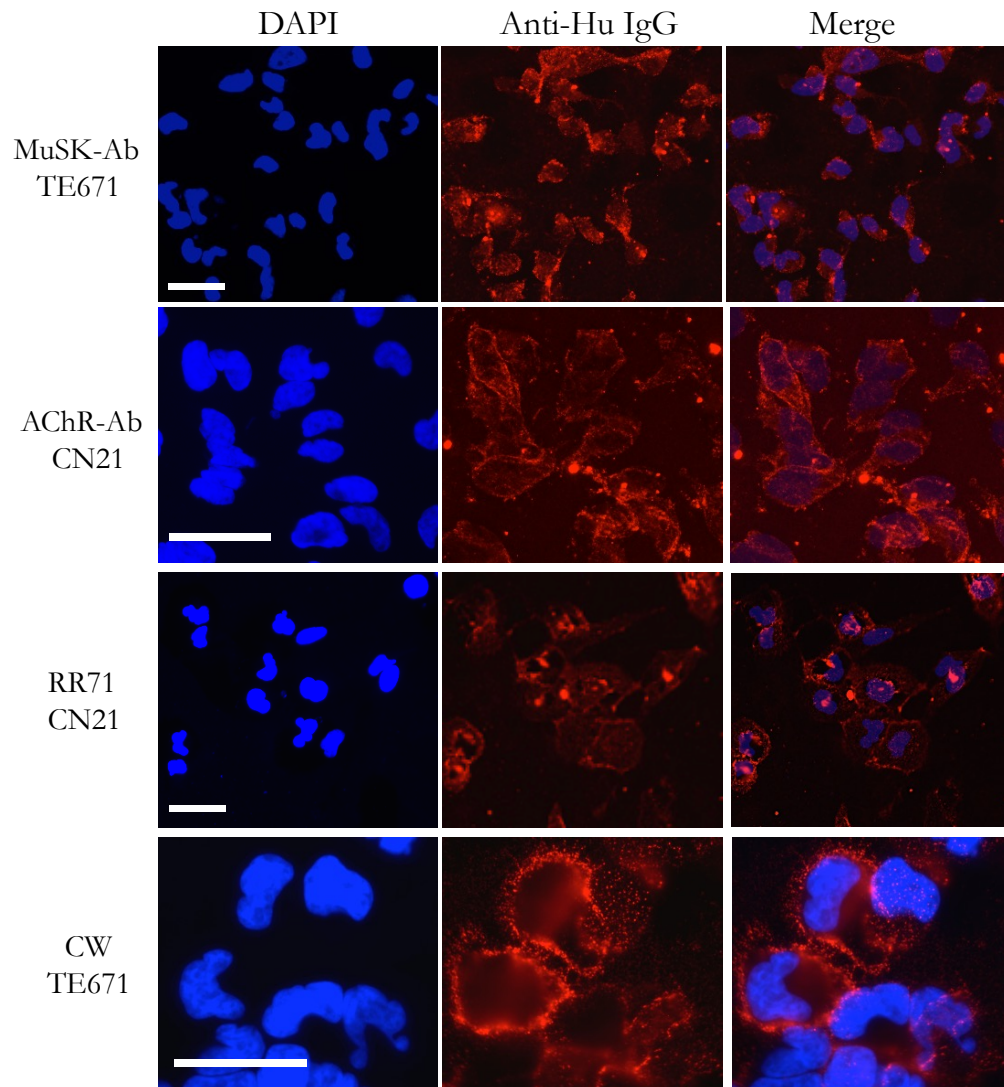


Figure 3.18. TE671/CN21 cell-based assays- Sera (1:10) were incubated with live unpermeabilised TE671 and CN21 cells for one hour at room temperature, and bound IgGs detected with Alexa Flour® 568 anti-human IgG (heavy and light chains; red). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Representative images- *Upper*: MuSK-MG control sera binding to TE671 cells. Magnification 40x; scale bar = 50 μm. 2nd row: AChR-MG control sera binding to CN21 cells. Magnification 100x; scale bar = 20 μm. 3rd row: triple-SNMG sera (RR71) binding to CN21 cells. Magnification 40x; scale bar = 50 μm. *Lower*: triple-SNMG sera (CW) binding to TE671 cells. Original magnification 100x (image cropped); scale bar 20 μm

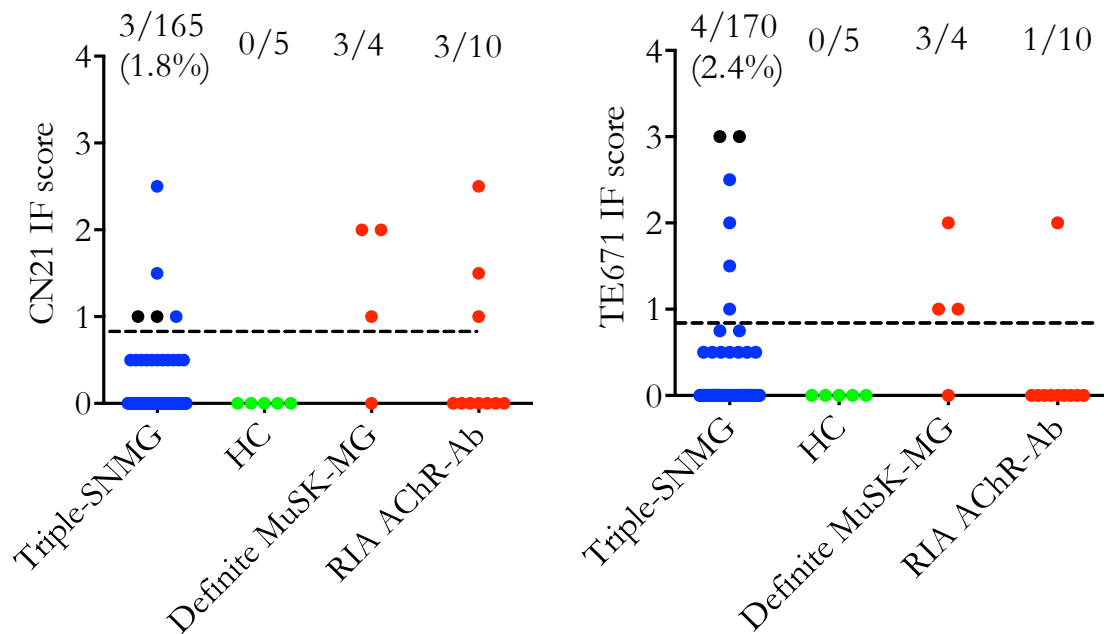


Figure 3.19. Scatter plots of patient and control sera using TE671/CN21 cell-based assays- Triple-SNMG sera labelled in black bound HEK-cells non-specifically and were designated negative. IF = immunofluorescence.

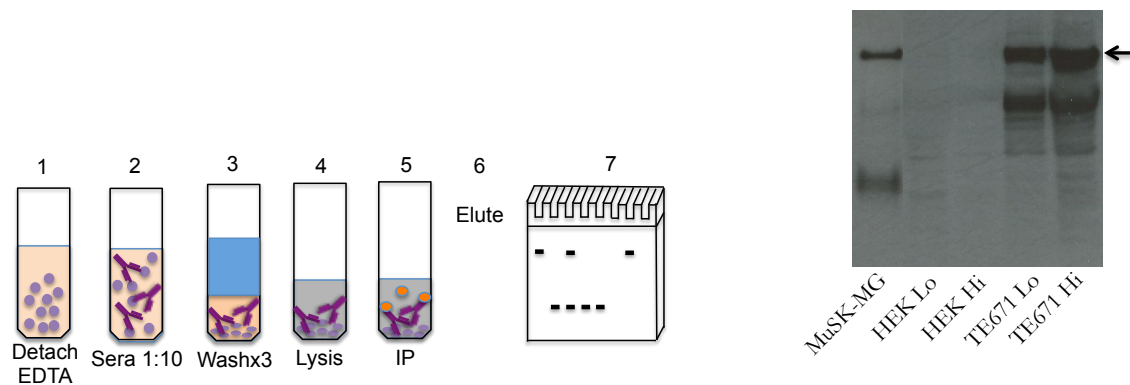


Figure 3.20. *Left:* Immunoprecipitation- TE671/CN21 cells were detached using EDTA and incubated with sera (1:10). Cells were then washed, lysed, and antigen bound to IgGs immunoprecipitated with protein G. Following elution the proteins were separated by SDS-gel electrophoresis and probed for MuSK by Western blot. *Right:* Representative western blot with anti-MuSK commercial antibody (R+D systems; AF562)- A 97kDa band (signified by arrow) is seen in positive control lanes (TE671 low and high) and with an immunoprecipitate with a MuSK-MG control sera, but not in negative controlled lanes (HEK low and high).

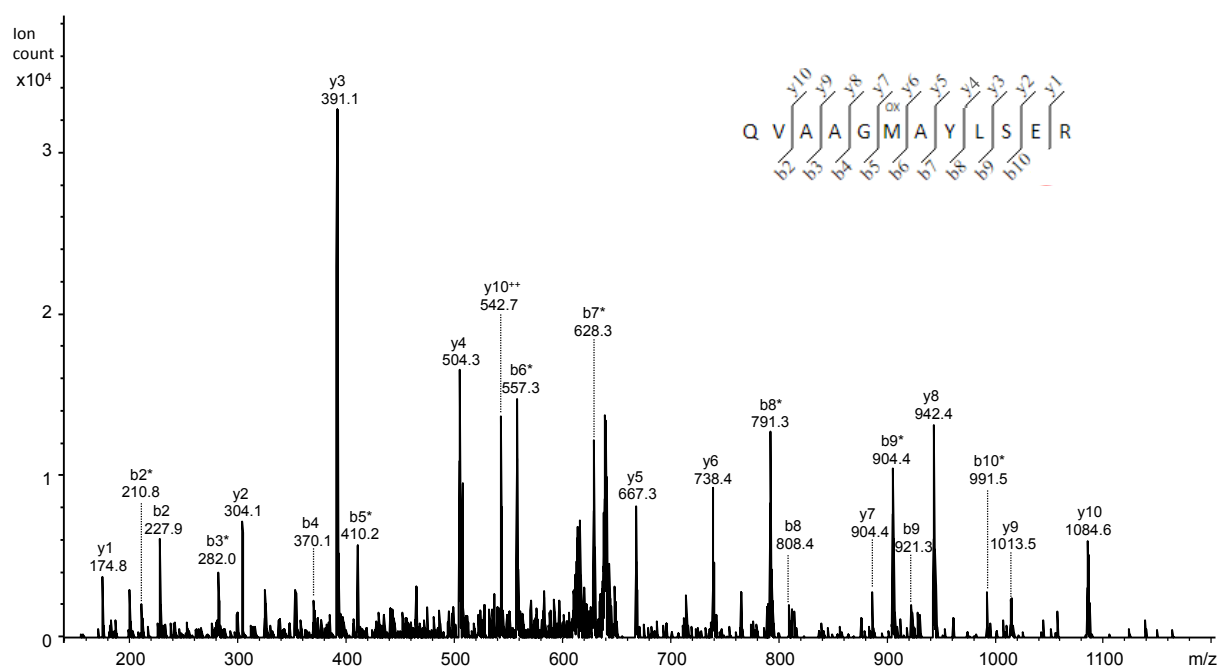


Figure 3.21. Peptide sequences identified by tandem mass spectrometry correspond to MuSK- 3 sequences specific for MuSK were identified from the MuSK-MG immunoprecipitate; MS/MS fragmentation spectrum of MuSK tryptic peptide 708-719 identified with a Mascot score of 80.

The experiment was repeated but following SDS-PAGE the gel was stained with Coomassie blue. In-gel digestion was performed followed by liquid chromatography and tandem mass spectrometry (MS/MS) using a 3D high capacity ion trap mass spectrometer. For full details please see the Chapter 2 : Materials and Methods. **Figure 3.21** demonstrates the fragmentation ion spectra of the MuSK tryptic peptide 708-719, which was detected with a MASCOT score of 80 (scores ≥ 35 were considered significant with $p < 0.05$). Two further significant peptides were also identified in this experiment.

Following optimisation of the IP and LC-MS/MS protocol the 3 triple-SNMG sera positive with the TE671/CN21 CBA and the MuSK-MG control serum were tested. Each sample was run in parallel with a HC serum to allow recognition and subsequent exclusion of proteins that were captured non-specifically. MuSK was identified on each run and the results of the extracellular and secreted proteins uniquely identified in triple-SNMG sera are summarised in **Table 3.2**. Unfortunately none of the cell surface or secreted proteins were plausible target antigens after filtering results based on expression and functional relevance to neuromuscular transmission. In addition none of the proteins listed were commonly identified between the MG patients.

Interestingly, several proteins of the survival motor neuron complex (SMN; intracellular) were uniquely identified (Gem-associated protein 2/4/5/6 and survival motor neuron protein; top MASCOT score- 331; Gem-associated protein 5) in the immunoprecipitate of 'CW'. This patient had polymyositis in addition to MG, and antibodies against the SMN complex have previously been described in patients with polymyositis (Sato et al. 2011).

Protein	Location	NMJ expression	Function/association	Disease association
Apolipoprotein(a)	E	no	lipoprotein component	pulmonary fibrosis
G-protein coupled receptor 182	E	no	orphan G-coupled receptor	-
Glycerophosphodiester phosphodiesterase domain-containing protein 3	E	no/unk	Glycerophosphodiester phosphodiesterase activity	-
Olfactory receptor 2AJ1	E	no/unk	olfaction	-
P2Y purinoceptor 6	E	no/unk	G-protein activated UDP-> UTP ->ATP	-
PDZK1-interacting protein 1	E	no	Renal proximal tubular epithelial cells	-
Protocadherin-7	E	no	cell-cell recognition and adhesion	-
Protogenin	E	yes	anteroposterior axis elongation*	-
Sodium channel protein type 3 subunit alpha	E	no	voltage-dependent sodium ion permeability	-
Sodium-dependent neutral amino acid transporter B(0)AT2	E	no	sodium dependent neutral amino acid transporter	-
Solute carrier family 22 member 4	E	no	carnitine transporter	RA
Thrombospondin type-1 domain-containing protein 7B	E	no	not characterised	membranous nephropathy
Transferrin receptor protein 1	E	no	transferrin carrier protein	-
Tumor necrosis factor receptor superfamily member 16	E	no	glucose uptake	-
Endothelin-converting enzyme-like 1	I/E	unk	peptide hormones degradation	distal arthrogryposis
ATP synthase subunit alpha	I/E	yes	ADP->ATP	COPD 22
Alpha-2-macroglobulin	SEC	no	antiprotease, inhibits coagulation	Amyloid, SLE, APS
Apolipoprotein A-1	SEC	no	cholesterol transport	-
Brain-specific serine protease 4	SEC	no	serine protease	-
C-C motif chemokine 27	SEC	no/unk	chemotaxis	-
C4b-binding protein alpha chain	SEC	no/unk	regulates complement activation	-
Collagen alpha-2(IV) chain	SEC	yes	component of glomerular basement membrane	Porencephaly
Complement C1q subcomponent subunit C	SEC	no	complement activation	C1q deficiency
Emilin	SEC	yes	extracellular matrix constituent	-
Fibrinogen gamma chain	SEC	no	fibrin matrix formation, coagulation	coagulopathy
Otogelin-like protein	SEC	no	inner ear function	deafness
Otolin-1	SEC	unk	otolith crystal anchoring	-
Serum albumin	SEC	no	plasma protein	HFD
Serum amyloid P-component	SEC	no	component of amyloid	-
Uromodulin	SEC	no	apical membrane of loop of Henle	familial JHN

Table 3.2. Summary of extracellular and secreted proteins uniquely identified in immunoprecipitates of triple seronegative MG sera. E = extracellular; SEC = secreted; I = intracellular; UDP = uridine diphosphate; UTP = uridine triphosphate; RA = rheumatoid arthritis; COPD22 = combined oxidative phosphorylation deficiency

22; SLE = systemic lupus erythematosus; APS = antiphospholipid syndrome; HFD = hyperthyroxinaemia familial dysalbuminaemic; JHN = juvenile hyperuricaemic nephropathy.

3.4 Discussion

The prevalence of AChR-, MuSK- and LRP4-Abs has been determined in 250 MG patients from European, African, and Asian countries. CBAs improved the detection of antibodies in 18% of patients negative by radioimmunoassays; clustered AChR-Abs (10%), MuSK-Abs (7%), and LRP4-Abs (1%). Clustered AChR-Ab positive patients had a poorer prognosis than has previously been noted with a paediatric cohort (18% vs. 50%; Rodriguez Cruz et al. 2015). Initially poor sensitivity and specificity of the MuSK CBA was noted. This assay was optimised through use of an IgG specific secondary antibody. Sera containing MuSK-IgM frequently resulted in false positives, were detected in MG and HC sera alike. Despite the use of CBAs, the majority of patients (76%) were designated, triple-seronegative MG. The clinical course of patients in this group with generalised disease was often severe (MGFA>2) and remission rates were only 18%. Interestingly 19% of triple-SNMG sera tested reduced AChR clustering in cultured myotubes. To determine if triple-SNMG sera contained antibodies against novel antigenic targets, sera were assessed for binding to TE671 and CN21 muscle cell lines. Three triple-SNMG were identified but disappointingly immunoprecipitation and mass spectrometry failed to identify novel antigenic targets. Modification of this methodology is likely to be required to identify successfully novel antigens in MG.

Using RIAs, approximately 80% of generalised MG and 50% of ocular MG have detectable AChR antibodies. False positives are infrequent (Vincent & Newsom-Davis 1985; Lindstrom et al. 1976) but were surprisingly noted in 6% of sera. The majority of these patients were from South Africa. Higher false-positives rates in African populations have been reported with other diagnostic assays such as with human immunodeficiency virus testing, and it is presumed that this is secondary to parasitic exposure and polyclonal B-cell activation (Klarkowski et al. 2014). AChR-RIA false positives have also been reported in patients with Lambert-Eaton myasthenic syndrome and asymptomatic family members of

MG patients but they are rare (Somnier 1993). One explanation that was not investigated was whether these sera immunoprecipitated iodinated alpha-bungaratoxin. In one study antibodies against alpha-bungaratoxin were found in 13% of MND sera, and interestingly many of the patients had prior exposure to snake venom-based treatments (Mittag & Caroscio 1980).

Clustered AChR-Abs were detected in 10% of MG sera, much lower than the reported rates from two other studies (38%; Rodriguez Cruz et al. 2015; 66%; Leite et al. 2008), but similar to a French study (16%; Devic et al. 2014). One reason for this may have been due to differences in patient selection. Most of the patients included in the study by Rodriguez-Cruz et al. were patients under the age of 18 yrs (Rodriguez-Cruz et al. 2015). The study by Leite et al. examined sera from patients with generalised MG only. It is logical to presume that the higher rates AChR-Ab detection by RIA in generalised MG as compared to ocular MG also apply to detection of AChR-Abs by CBAs (Leite et al. 2008). In addition many samples studied here were obtained after immunotherapies, and archived sera stored at -20°C may have degraded over time and with prior freeze thaw cycles.

Almost half of the patients with clustered AChR-Abs (47%) had ocular MG, but 40% reached a peak MGFA>2. High response rates to acetylcholinesterase inhibitors (83%) and immunotherapy (93%) were noted but remission rates were only 18%. This was considerably lower than the rate of 50% we previously reported in clustered AChR-MG patients under 18 years old (Rodriguez-Cruz et al. 2015) and suggests a poorer prognosis for adults with clustered AChR antibodies. One patient was found to have antibodies against the foetal isoform of AChR. Foetal or extrajunctional AChRs ($\alpha:\beta:\delta:\gamma$; 2:1:1:1) are replaced by the adult or junctional AChR ($\alpha:\beta:\delta:\epsilon$, 2:1:1:1) isoform *in utero*, but continue to be expressed in ocular muscles. The presence of foetal specific AChR-Abs is of particular importance in expectant mothers where the Abs can cross the placenta and cause neonatal MG, arthrogryposis multiplex congenital, and foetal AChR inactivation syndrome (Polizzi et

al. 2000; Hacohen et al. 2015). As the patient identified was female and of child-bearing age, the responsible clinician was informed. Most patients have AChR-Abs that bind to both isoforms, as the α -subunit is common to both, but it would have been of interest to assess all MG sera, particularly female patients for the presence of foetal specific AChR-Abs, which are associated with these foetal syndromes.

The detection of bound IgM resulted in poor disease specificity in the MuSK-CBA. Using an unlabeled secondary antibody specific for the Fc(γ) chain of IgG, and applying a tertiary fluorescent antibody for detection, MuSK-Ab detection was increased in MuSK-MG sera, whilst avoiding detection of the non-specific IgM bound to MuSK, which had initially occurred in 11% and 19% of healthy and disease control samples respectively. Overall, the IgG-specific MuSK-CBA detected MuSK-Abs in 7% of sera negative by MuSK-RIA. The IgM bound to MuSK in MG sera was specific for MuSK by colocalisation and preadsorption experiments. IgM is normally in the form of pentamers with up to ten binding sites. One possible explanation for IgM detection in these assays could be the non-antigen specific binding of IgM pentamers to certain types of protein expressed at high levels on cells.

LRP4-Abs were only detected in 1% of patients, which is not dissimilar to rates reported in other studies (3%; Higuchi et al. 2011 and 9.2%; Zhang et al. 2012). Detection rates of 18.7% (Zisimopoulou et al. 2014) and 54% (Pevzner et al. 2012) have also been reported, and the wide variability may have in part been due to the different assay techniques (luciferase-reporter immunoprecipitation, CBA, and ELISA) that were used by the different research groups. A high proportion of LRP4 antibodies detected have been associated with other autoantibodies (Cossins et al. 2012), and of the three patients identified here, two had antibodies to other targets. One patient with mild ocular MG also had AChR antibodies, suggesting that the presence of LRP4 antibodies did not confer a more severe clinical phenotype.

Agrin and Col-Q antibodies have also been reported in MG. Agrin antibodies have been shown to inhibit agrin dependent MuSK phosphorylation and AChR clustering. However, like Col-Q antibodies they are rarely detected in isolation, and as such their relevance to disease is less clear (Gasperi et al. 2014; Zhang et al. 2014; Cossins et al. 2012; Zoltowska et al. 2015). Here it was found that Agrin and Col-Q CBAs were frequently positive in healthy control sera and these assays were not pursued further.

Overall, despite the use of RIA/CBAs, 76% of MG sera were negative for AChR-, MuSK- and LRP4-Abs. Previous studies have suggested a mild phenotype for patients negative by AChR- and MuSK-RIAs (Oh et al. 2012; Romi et al. 2005). This was the case with male patients who had a less severe nadir of disease than females, perhaps due a higher proportion of ocular MG in this group (30%). However, 50% of triple-SNMG patients reached a MGFA>2, suggesting in the generalised MG cases the clinical course was not mild. Although only 57% of triple-SNMG patients had supportive neurophysiology, the diagnosis of MG appeared accurate based on characteristic clinical features as determined by MG specialists and a reassuringly high response rate (~80%) to acetylcholinesterase inhibitors and/or immunotherapies. However, only 18% of triple-SNMG patients achieved remission following treatment. These results suggest that the poorer prognosis that has been noted in a larger South African cohort also applies to triple-SNMG in Europe and Asia, albeit some of those South African patients were also included in this analysis (Huda et al. 2016). Importantly many of these patients had a bulbar predominant phenotype, and it is likely there would have been selection bias from other cohorts also. The clinical analysis was also limited by the retrospective design and lack of complete clinical data in all of the patients.

To further study the triple-SNMG cohort, sera were tested for effects on AChR clustering. Interestingly 19% (3/16) of sera tested inhibited agrin induced AChR clustering. This may suggest that some triple-SNMG sera have effects on the AChR clustering pathway, but experiments to determine if this finding was due to reduced surface AChRs could not be performed due to limited volumes of sera available. To screen triple-SNMG for novel

antigenic targets a CBA using TE671/CN21 muscle cell lines was established. Only 21% and 75% of control AChR-MG and MuSK-MG control sera were positive by this method, but 3 triple-SNMG that bound to both CN21 and TE671 cells were identified. The sensitivity might have been improved through use of FACS, as had originally been reported (Blaes et al. 2000). Perhaps not unsurprisingly the mass spectrometry results yielded no plausible target antigens after filtering results based on proteins bound by HC sera, cell surface localisation, and expression at the neuromuscular junction. However, it would be of interest to determine the gene expression of respective proteins uniquely identified by triple-SNMG sera in muscle tissue. Many intracellular proteins were also detected in both disease and HC samples as has previously been noted (Littleton et al. 2009), and were likely secondary to non-specific capture during immunoprecipitation. It was also possible that prior to lysis, unbound Abs from patient sera were not sufficiently removed by washing, allowing for binding to intracellular targets that were also immunoprecipitated by protein G.

The low proportion and relatively weak binding of triple-SNMG sera and control sera to CN21/TE671 cells suggests that these cells may not express sufficient levels of post-synaptic NMJ cell surface proteins. The proteomic profile of TE671 cells has been studied using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry but possibly due to methodological differences, neuromuscular junction proteins including MuSK were not detected (Lenaerts et al. 2007).

In one sample several of the intracellular proteins identified were of interest as they were all components of the survival motor neuron (SMN) complex. This sample was from a patient with coexistent polymyositis, and antibodies against the SMN complex have been previously described in this condition (Sato et al. 2011). In addition, Tripartite motif-containing 21 (TRIM21), a cytosolic protein that binds to the CH2/CH3 interface of IgG with nanomolar affinity, was also identified. TRIM21 is involved in the intracellular arm of adaptive immunity; capturing antibody bound virus and transporting it to the proteasome (Lahouti & Christopher-Stine 2015; Vidarsson et al. 2014). Interestingly, proteins of the

ubiquitin-proteasome pathway were upregulated in a mouse model of myositis, and treatment with bortezomib (a proteasome inhibitor) improved muscle strength and inflammation (Rayavarapu et al. 2013). It is therefore possible that TRIM21 may facilitate antibody access to the proteasome in polymyositis. However, access to cytosolic antigens is restricted *in vivo* due to an intact cell membrane. Antibodies to the SMN complex may be analogous to those against other intracellular targets, and it is generally accepted that these Abs rather than being pathogenic, are markers of disease (Racanelli et al. 2011).

TE671/CN21 CBAs were performed prior to the realisation of cross-reactivity that anti-human IgG (heavy and light chains) cross-reacted with other Ig isotypes. Detection rates and IgG specificity may have been improved if the latter had been used. It would have also been ideal to have re-tested the three sera with positive binding to TE671/CN21 cells using anti-human IgG Fc(γ), but the volumes of sera available were suitable only for IP and LC-MS/MS experiments. However, overall the results suggest it may be beneficial to assess other cell lines for IP and MS experiments such as the immortalised human skeletal muscle cell line- HMCL-7304 or ideally human stem cell derived motor neurons and skeletal muscle co-cultures (Rokach et al. 2013; Guo et al. 2011).

Chapter 4: Clinical and serological features of myasthenia gravis with MuSK antibodies

4.1 Introduction

MuSK-MG accounts for between 3 - 47% of AChR-Ab negative MG (X. Zhang et al. 2007; Evoli et al. 2003). MuSK-Abs are routinely detected using a MuSK-RIA, and patients positive by this assay have a broadly similar phenotype. The patients are mainly young females with prominent bulbar involvement and frequent respiratory crises, particularly in the first two years. It has been suggested that within this broad phenotype there are three main categories of MuSK-MG - 1) severe faciopharyngeal weakness, 2) predominant neck and respiratory weakness, 3) clinical features indistinguishable from non-MuSK-Ab MG (non-MuSK-MG; Evoli et al. 2003; Guptill et al. 2011; Guptill & Sanders 2010).

The clinical phenotype of patients with MuSK-Abs detected solely by CBAs is unclear and has only been reported in one study to date (Tsonis et al. 2015). Two studies from Sri Lanka (Chang et al. 2014) and China (Yang et al. 2011) reported the detection of 6 MuSK CBA positive patients, but individual clinical details were not given.

In contrast to MuSK-MG patients, Tsonis et al. found that 27% of MuSK-CBA positive patients had ocular MG and 23% had thymic hyperplasia. In addition only 8% (2/25) of sera were positive for MuSK-IgG Abs, but all contained MuSK-IgM Abs. This result was surprising given the established predominance of MuSK-IgG4 Abs, for which a

pathogenic role has previously been established (Hoch et al. 2001; McConville et al. 2004; Huijbers et al. 2013; Koneczny et al. 2013; Klooster et al. 2012). Functional effects on AChRs have previously been noted with SNMG non-IgG fractions, and suggested to be IgM, but these effects were never confirmed to be caused by IgM-Abs (Barrett-Jolley et al. 1994).

As the MuSK-MG cohort was to be used for therapeutic studies in Chapter 5, the first aim of this chapter was to analyse the serological characteristics of these patients. This included antibody titres, subclass, and the relationship of these measures to clinical severity. The second aim was to compare the clinical features of MuSK-RIA and MuSK-CBA positive sera. The final aim was to assess pathogenic effects of MuSK-CBA positive sera and MuSK-IgM containing sera on AChR clustering in myotubes.

4.2 Clinical characteristics of MuSK-MG

MuSK-MG sera = 71, were sent from Pisa, Italy by the MG specialist Dr. Ricciardi. The diagnosis was established on the basis of characteristic clinical features, detection of MuSK-Abs (determined by RIA), and a beneficial response to immunotherapy. Neurophysiology was not routinely performed. The clinical features of MuSK-RIA patients are summarised in **Table A.2** in Appendix A.

Patients were predominantly female (M:F; 1:9) through the age range. The age of onset in patients was between the ages of less than 12 months old to 50yrs. with a median of 40 (1-79) yrs (**Figure 4.1**; left). A comparison of early onset (<40years) and late onset (≥ 50 yrs) MuSK-MG patients did not reveal differences in gender, disease course, antibody titres, or treatment outcomes (data not shown). There were only 7/71 (10%) male patients and they were similar in demographic and clinical features to female patients. There were no differences in the season in which patients were born, but when patients first noted symptoms was not evenly distributed throughout the seasons, and the direction appeared to

favour winter months (Nov-Feb, $p=0.04$; binomial test; **Figure 4.1**; right). Unfortunately information regarding potential triggers such as infection was not provided.

The median time from symptom onset to diagnosis was 1 (0-10) year. Oropharyngeal involvement was the most prominent symptom in 69/71 (97.2%) and, at first review, 49/67 (73.1%) had an MGFA>2. Four patients presented with respiratory failure requiring immediate intubation and ventilation. Ocular symptoms were present in 58/71 (81.7%) of patients and 16/71 (22.5%) had involvement of limb muscles. A family history of AChR-MG was noted in 4/71 (5.6%) patients and concurrent autoimmune disease in 12/71 (16.9%) of patients (most commonly thyroid disease). A 'Guptill phenotype' (Guptill & Sanders 2010) was available in 70 patients of which 28/70 (40%) had features that were indistinguishable from non-MuSK-MG, 27/70 (38.6%) had predominant faciopharyngeal involvement, and 15/70 (21.4%) patients had predominant neck extensor and respiratory weakness. Patients with predominant neck extensor and respiratory weakness had higher MGFA scores at presentation than those with features indistinguishable from non-MuSK-MG ($p=0.01$; Kruskal-Wallis test; **Figure 4.2**). Three typical patients are described-

Case 1. (non-MuSK-MG) M083, a 19-yr old female developed mild ptosis and diplopia, followed by limb weakness and dysphagia (MGFA 2B). Pharmacological remission was achieved and the patient remained on Prednisone 7.5mgs alternate days without the need for additional medication.

Case 2. (faciopharyngeal predominant) M147, a 33-yr old female developed difficulty swallowing with dysarthria and ptosis. Weakness of tongue movements without atrophy was noted. A thymectomy was performed without benefit. The patient has been in pharmacological remission on Prednisone 12.5mg/day.

Case 3. (predominant neck extensor and respiratory) M128, a 50-yr. old female presented with subacute progressive neck extensor, bulbar and respiratory muscle weakness (MGFA 4b). Her family history was notable for a maternal cousin with AChR-MG. She was commenced on steroids with an excellent response and was in pharmacological remission on

Prednisone 6.5mg/day. The individual clinical data of all the MuSK-MG positive patients are summarised and included within Appendix A.

4.3 Treatment and outcome

Immunotherapy, of which the mainstay was prednisone, was given in 69/71 (97.2%) of patients. Ten patients received azathioprine as a 'steroid-sparing' agent. IVIG rather than plasma exchange was the preferred rescue treatment and was required in 10/71 (14%) patients. Two patients also received plasma exchange at regular intervals. Immunotherapy was beneficial in 67/69 (97.1%) of patients. At follow-up 11/70 (15.7%) of patients had an MGFA>2 compared to 49/67 (71.3%) at onset ($p<0.0001$; Fisher's test); (median follow-up- 9 (2-34) years). Accordingly a PIS of minimal manifestations/remission was attained in 40/67 (59.7%) and pharmacological/complete stable remission in 26/69 (37.7%) of patients. There were no differences in rates of remission between different MuSK-MG phenotypes (data not shown). Thymectomy was performed in 6/71 (9%) of patients. Histological features were normal in 3/6 and suggestive of thymic hyperplasia in the remaining three samples. Post-operative improvement was not noted in any of these patients.

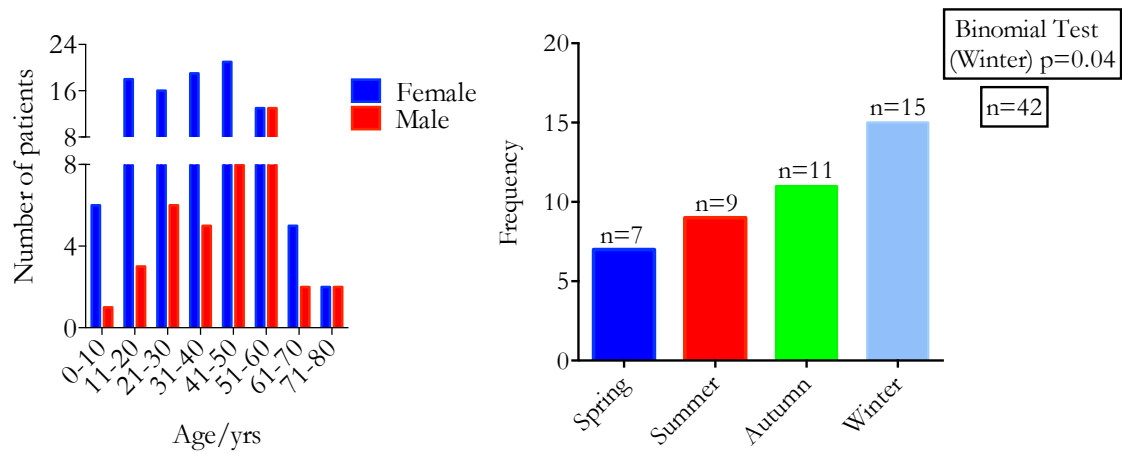


Figure 4.1. *Left:* Frequency distribution of age and sex in 71 MuSK-MG patients. *Right:* Frequency distribution of season of symptom onset in 42 MuSK-MG patients ($p=0.04$; Binomial test).

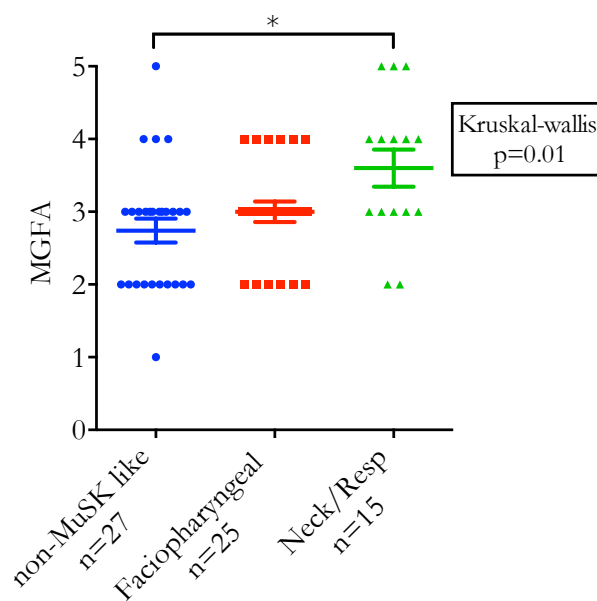


Figure 4.2. Guptill phenotype and MGFA at presentation in 67 MuSK-MG patients- Patients with predominant neck and respiratory weakness were more likely to present with higher MGFA grades than patients with features indistinguishable from non-MuSK-MG ($p=0.01$; Kruskal-Wallis).

4.4 Serological characteristics

As shown in **Figure 4.3**, 66/71 (93%) of patients had samples taken following exposure to immunotherapy when they had lower MGFA grades compared to disease onset. The MuSK-Ab status of the patients was first re-checked by MuSK-RIA with sera diluted 1:10. MuSK-Abs were detected in 69/71 (97.2%) samples. To determine more precisely the titre of MuSK-Abs the concentration of MuSK-Abs, which no longer saturated radiolabelled MuSK was determined. An example of MuSK-Ab titre determination is shown in **Figure 4.4** (left) and a summary of these data are shown in **Figure 4.4** (right). There was weak correlation between MuSK-Ab/nM and MGFA at sampling ($p=0.01$ $r^2=0.09$; Pearson's test) suggesting that the concentration of MuSK-Abs was broadly related to disease severity.

MuSK-MG sera were subsequently tested by CBAs. As described in Chapter 3, MuSK-Abs were initially detected in only 54/71 (76%) patients using a secondary antibody against heavy and light chains of human IgG. Following substitution of this antibody with anti-human IgG Fc(γ), MuSK-Abs were detected in 70/71 (98.6%). M107 and M136 were both negative by MuSK-RIA (0.03 and 0.01 nM respectively) but positive by CBA (CBA scores 1 and 1.5 respectively). M145 bound non-specifically to HEK cells and was designated negative. The presence of other antibodies was assessed by AChR-RIA, clustered AChR and LRP4 CBAs. Low RIA AChR-Ab titres were detected in three MuSK-MG sera (0.55-0.73 nmol/L) that were negative for clustered AChR-Abs. Clustered AChR-Abs were detected in M154 and LRP4-Abs in M085, M133, and M178 (**Figure 4.5**).

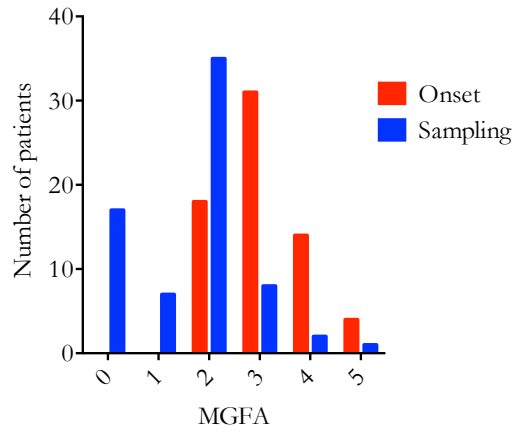


Figure 4.3. MGFA status of 67 MuSK-MG patients at disease onset and 70 MuSK-MG at time of serum sampling.

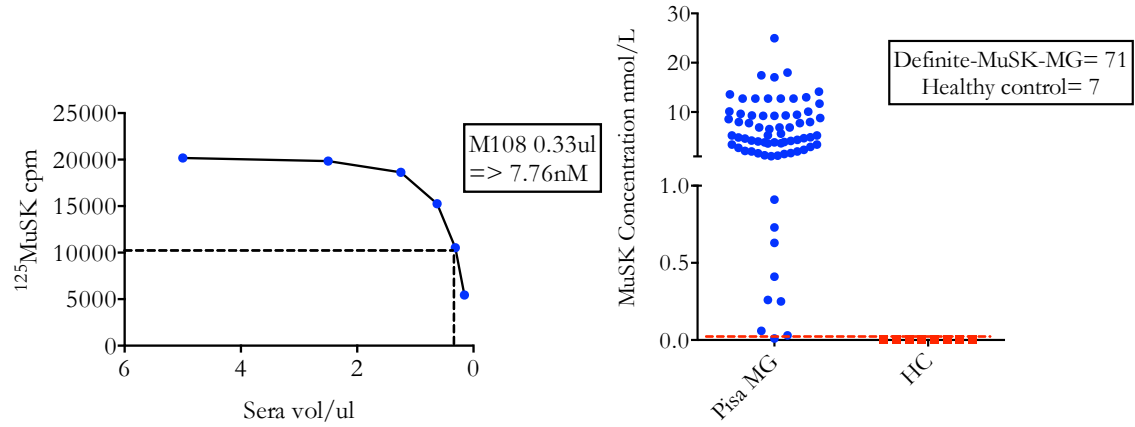


Figure 4.4. *Left:* Determination of MuSK-Ab titre- Serial dilutions of 71 MuSK-MG sera were tested by radioimmunoassay, and the resultant ^{125}I -MuSK cpm counts were plotted against the respective volumes of sera used. The concentration of MuSK-Abs, which no longer saturated radiolabelled MuSK was then extrapolated. *Right:* Summary of MuSK-Ab titre/nM results of MuSK-MG sera.

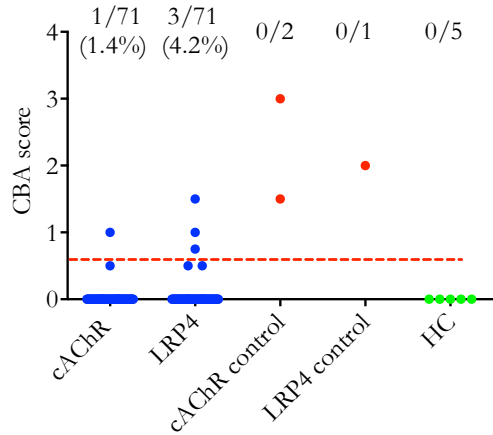


Figure 4.5. Scatter plots of clustered-AChR and LRP4 cell-based assay testing in MuSK-RIA positive and control sera

To assess the predominant MuSK-IgG subclass, end-point titres of MuSK-IgG and IgG1-4 were determined at the dilution prior to which MuSK-Abs were no longer visible by immunofluorescence. **Figure 4.6** shows the reduction of anti-human IgG (red) binding to MuSK transfected HEK cells (green) with serial dilutions of a MuSK-RIA positive sera and **Table A.2** in **Appendix A** contains the end-point titre data of all the patients.

As expected endpoint titres of MuSK-IgG were higher than for respective IgG subclasses. In most cases, MuSK-IgG4 end-point titres were higher than MuSK-IgG1-3 but MuSK-IgG1-3 Abs were frequently detected (IgG4 vs. IgG1-3; $p < 0.001$, Wilcoxon matched pairs signed rank test; **Figure 4.7**; left). Unexpectedly MuSK-IgG1-3 Ab titres were higher than MuSK-IgG4 Ab titres in 20/70 (29%) of MuSK-MG sera. Positive correlation was observed between MuSK-IgG4 and MuSK-IgG scores but not other subclasses (IgG1; $r = -0.01$ $p = 0.92$, IgG2; $r = -0.27$ $p = 0.03$, IgG3; $r = -0.32$ $p = 0.08$, IgG4 $r = 0.8$ $p < 0.0001$; Spearman's test) suggesting the majority of fluorescent signal in the optimised MuSK CBA was from MuSK-IgG4 Abs (**Figure 4.7**; right). MuSK-IgG2 and IgG4 broadly correlated with MGFA at sampling (IgG2; $r = 0.26$ $p = 0.03$ and IgG4 $r = 0.26$, $p = 0.03$; Spearman's test; data not shown). There were no observable differences in distribution of IgG subclass end-point titres amongst different MGFA grades or phenotypes (**Figure 4.8**; left and right), but on visual inspection higher MGFA grades were associated with higher MuSK-IgG4 end-point titres (**Figure 4.8**; left). MuSK CBAs were also performed using anti-human IgM, and MuSK-IgM Abs were detected in 8/71 (11.3%) of patients (MGFA 0-2). Reassuringly, end-point titres of MuSK-IgG by CBA correlated with MuSK-Ab titres ($r = 0.89$; $p < 0.0001$; Spearman's test; **Figure 4.9**; left). Using an arbitrary cut-off of MuSK-IgG4 1:100 and MuSK-Ab 5nM, 'low' IgG4 and MuSK-Ab titres were associated with a higher proportion of patients who had attained remission ($p = 0.01$ and $p = 0.03$ respectively; Fisher's test; **Figure 4.9**; right). This appeared independent of steroid dosage, which was similar between high and low MuSK-IgG4 and MuSK-Ab titre groups ($p = 0.17$; Mann-Witney test; data not shown).

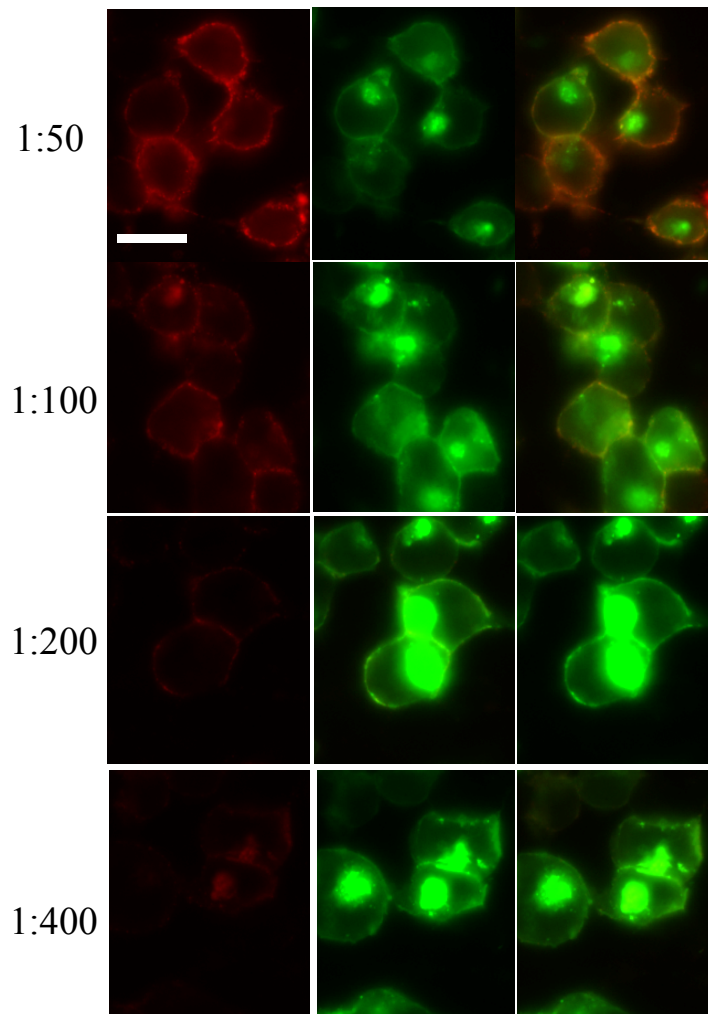


Figure 4.6. Serial dilution of a MuSK-MG sera on the MuSK cell-based assay (at higher dilutions the patients IgGs are only visible on a red filter). Magnification 100x; scale bar 20 μ M

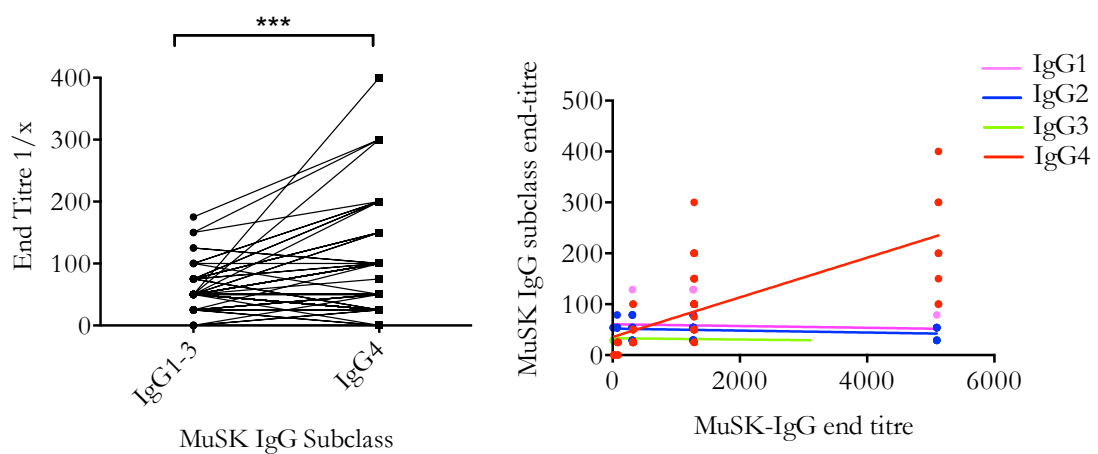


Figure 4.7. *Left:* End-point titres of MuSK-IgG4 were higher than MuSK-IgG1-3 ($p=0.0002$; Wilcoxon test) in 70 MuSK-MG patients. *Right:* Correlation was noted between MuSK-IgG and MuSK-IgG4 end-point titres ($r=0.8$ $p<0.0001$; Spearman test)

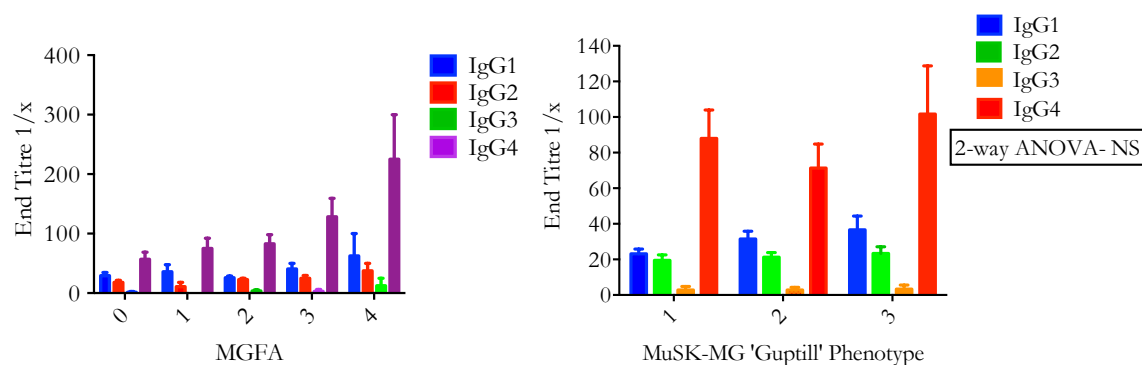


Figure 4.8. *Left:* MuSK-IgG subclass end-titre and MGFA grade in 70 MuSK-MG sera. *Right:* IgG subclass end-point titre and MuSK-MG 'Guptill' phenotype ($p > 0.05$; 2-way ANOVA). 1 = non-MuSK-MG; 2 = faciopharyngeal; 3 = predominant neck extensor and respiratory muscle weakness.

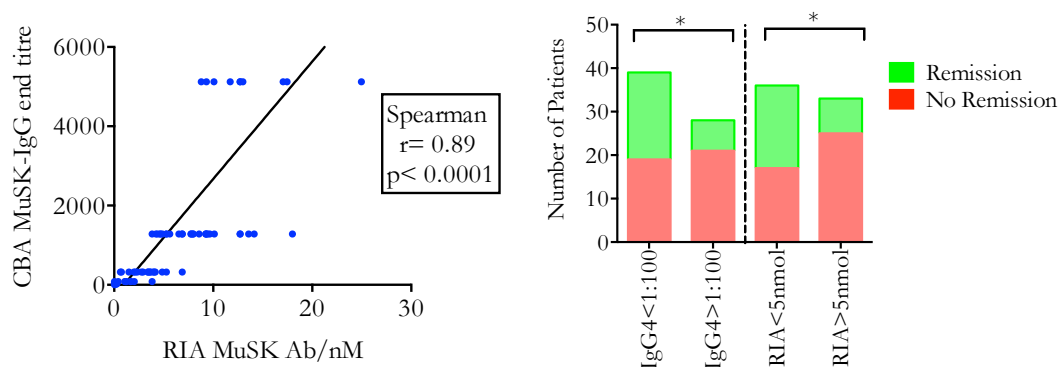


Figure 4.9. *Left:* Correlation between MuSK-IgG end-point titre and MuSK-Ab titre/nM (measured by radioimmunoassay); ($r = 0.89$ $p < 0.0001$; Spearman test). *Right:* IgG4 end-point titres < 1:100 (20/39 v.s 7/28) and MuSK-Ab < 5nM (19/36 v.s 8/33) were associated with remission ($p = 0.01$ and $p = 0.03$ respectively; Fisher's test)

4.5 Comparison of MuSK-IgG CBA and RIA Phenotypes

The country of origin and individual clinical data of MuSK-IgG CBA positive (RIA-negative) patients are summarized in **Table 4.1**. Patients were predominantly female (3.3:1) and presented at a median age of 25 years (range 16-79). At presentation to the neurologists, eight patients had disease confined to the ocular muscles (median duration of follow-up 2yrs (1-4.5)) and Myasthenia Gravis Foundation America (MGFA) grades were ≤ 2 in 11/13 (85%) patients. Patients were treated with therapies including acetyl cholinesterase inhibitors (AChEIs) only, or combinations with prednisone, azathioprine, cyclophosphamide, or rituximab. Post-intervention status suggested a favorable response to immunotherapy in all patients and remission was achieved in 3/11 (27%) patients.

Table 4.2 compares the clinical features of MuSK-IgG CBA positive with MuSK-MG patients. A female preponderance was noted in both groups (3.3:1 vs. 9:1; $p=0.1816$; Fisher's test) but the median age of onset was later in MuSK-MG patients (40 yrs (12-82) compared with 25 yrs (16-79); $p=0.01$; Mann-Whitney test). Generalised MG was reported in all 69 MuSK-MG sera patients, compared with 8/13 of MuSK-IgG CBA positive patients ($p<0.0001$; Fisher's test), with only 18/67 (27%) MGFA ≤ 2 at onset ($p=0.0001$; Fisher's test). Neurophysiology was only positive in 3/6 examined MuSK-IgG CBA patients. Reflecting the relatively mild phenotypes, AChEI was effective in 75% of MuSK-IgG CBA patients, compared with only 20% of MuSK-MG patients but, importantly, immunotherapies appeared equally effective in both groups, and final disease status did not differ, although follow-up times were clearly different.

MuSK-IgG CBA Positive/ country	Sex	Age of onset	MGFA onset	MGFA Max	MGFA last visit	Treatment	AChEI (+)	PIS
Patient 1 Germany	F	42	IIa	IIa	IIa	P	-	I
Patient 2 Germany	F	19	1	IIa	0	ACHEI, P, A	-	PR
Patient 3 Philippines	M	21	1	1	1	ChI, P	yes	-
Patient 5 UK	F	41	IIIb	IIIb	IIa	previous ACHEI, now P and MMF	yes	MM
Patient 6 Italy	F	16	I	IIb	IIb	ACHEI, previous P	no	MM
Patient 7 Norway	F	21	I	I	0	ACHEI	-	PR
Patient 8 Norway	M	79	I	I	I	ACHEI, P	-	-
Patient 9 Norway	F	20	I	I	I	ACHEI	yes	MM
Patient 10 Norway	F	27	I	I	0	ACHEI, previous P and A, then Ritux	-	CSR
Patient 11 South Africa	F	37	IVb	IVb	IVb	prev Cyclph, A, IVIG now Cyclosp,P	yes	I
Patient 12 South Africa	F	31	IIa	IIa	IIa	A, P	yes	MM
Patient 13 South Africa	M	25	IIa	IIa	1	MTX, P	no	MM
Patient 14 South Africa	F	25	1	V	IIIa	A, previous PLEX and IVIG	yes	MM

Table 4.1: Clinical data from 13 MuSK-IgG CBA positive patients. F = female; M = male; MGFA = Myasthenia Gravis Foundation America; P = prednisone; AChEI = cholinesterase inhibitor; A = azathioprine; Ritux = Rituximab; Cyclph = cyclophosphamide; Cyclosp = cyclosporine; PIS = post-intervention status; I = improved; MM = minimal manifestations; PR = pharmacological remission; CSR = complete stable remission, - = data not available. Note Patient 4 had insufficient clinical data and has been omitted from both Tables.

Parameter	MuSK-IgG CBA positive (n=13)	MuSK-MG (n=69)	<i>p</i> -value
Female: male	3.3:1	9:1	0.1816
Age at onset median (range)	25 (16-79)	40 (12-82)	0.0105 ¶
Generalised: ocular	8:5	69:0	<0.0001
MGFA onset ≤2	11/13 (84.6%)	18/67 (26.9%)	0.0001
MGFA follow-up ≤2	11/13 (84.6%)	58/69 (84.1%)	1.000
Neurophysiology positive	3/6 (50%)	-	-
Treatment			
AChEI response (+)	6/8 (75%)	14/69 (20%)	0.0032
Immunotherapy response	11/11 (100%)	67/69 (97%)	1.000
Post intervention status No. with remission	3/11 (27.3%)	26/69 (37.7%)	0.7376
Median follow-up/yrs.	2 (1-4.5)	8 (2-31)	<0.0001

Table 4.2. Comparison between 13 MuSK-IgG CBA positive and 69 MuSK-RIA positive patients. Unless otherwise specified all statistics were performed with Fisher's exact test. ¶: Mann-Whitney test

4.6 MuSK-MG and inhibition of the agrin/LRP4/MuSK/DOK7 pathway

Previous experiments have demonstrated the reduction of agrin-induced AChR clusters by MuSK-MG sera (Farrugia et al. 2007; Hoch et al. 2001; Koneczny et al. 2013), but it is not clear if these effects are related to MuSK-Ab titres or disease severity. To address these points, serial dilutions of MuSK-MG sera (1:10, 1:20, 1:40, 1:80, 1:160, 1:320) were applied to mouse C2C12 myotubes with agrin, and the resultant AChR clusters/microscopic field were determined. MuSK-MG sera were selected such that a broad range of MGFA and MuSK-RIA Ab titres/nM were included. These data and the AChR clustering results are presented in **Table 4.3**. As illustrated in **Figure 4.10**, there was an inhibitory effect of MuSK-Abs on agrin-induced AChR clustering that was concentration dependent. Broad correlation was noted between mean AChR cluster number and MuSK-IgG, but only if the outlier M97 (IgG end-titre 1/20480) was excluded ($p=0.03$, $r^2=0.53$ v.s. $p=0.25$, $r^2=0.43$; Pearson's test). MuSK-IgG4 ($p=0.04$; $r^2=0.43$; Pearson's test) and MGFA ($p=0.04$; $r^2=0.45$; Pearson's test), but not MuSK-IgG1-3 ($p=0.10$; $r^2=0.31$; Pearson's test) also demonstrated broad correlation with mean AChR cluster number (**Figure 4.11**).

4.7 Functional relevance of IgG and IgM MuSK antibodies

MuSK-MG sera inhibit the agrin/LRP4/MuSK/DOK7 pathway (Hoch et al. 2001; Huijbers et al. 2013; Koneczny et al. 2013). To determine if similar effects occurred with sera positive for IgG-MuSK-Abs and MuSK-IgM binding sera, AChR clustering assays were performed. All 6 MG sera with IgG-MuSK Abs, but not the 8 with only IgM non-specific MuSK-Abs, reduced the number of agrin-induced AChR clusters on C2C12 myotubes suggesting that only the IgG-specific Abs had pathogenic potential (**Figure 4.12**). Nevertheless, they were clearly less effective than the two MuSK-MG control samples tested in parallel that abolished

AChR clusters. Unfortunately due to the limited amounts of sera, experiments to assess the IgG subclass of sera with MuSK-IgG Abs could not be performed.

Sample	MuSK-RIA titre/nM	MGFA Sample	IgG1-3/IgG4 end-titre	IgG end-titre	1:10	1:20	1:40	1:80	1:160	1:320
Mean AChR clusters/microscopic field (% HC)										
M95	1.15	0	25/ 50	80	82.1 (108.0)	97.5 (121.2)	95.2 (108.3)	102.4 (121.6)	90.6 (101.4)	105.9 (123.4)
M163	2.00	2B	25/ 25	320	37.8 (49.7)	54.6 (67.9)	46.4 (52.7)	50.8 (60.3)	70.7 (79.2)	60.2 (70.2)
M113	2.03	2B	50/ 25	80	81.3 (107.0)	80.4 (99.9)	92.0 (104.6)	90.7 (107.8)	92.9 (104.0)	85.9 (100.1)
M153	2.92	2B	25/ 25	320	17.6 (23.1)	33.8 (42.0)	40.2 (45.7)	41.7 (49.6)	35.2 (39.4)	59.5 (69.4)
M108	7.76	0	50/ 50	1280	7.15 (6.31)	26.6 (33.1)	68.8 (78.3)	85.3 (101.5)	97.8 (109.5)	93.8 (109.3)
M91	8.80	2B	75/ 200	5120	12.5 (7.04)	14.6 (18.0)	18.3 (20.8)	36.7 (43.6)	45.8 (51.2)	51.3 (59.7)
M167	9.45	3B	75/ 100	1280	11 (6.58)	8.85 (11.0)	28.6 (32.5)	63.8 (75.8)	59.8 (66.9)	67.5 (78.8)
M93	12.7	3B	75/ 150	5120	7.15 (9.41)	6.40 (7.96)	23.2 (26.3)	37.2 (44.2)	64.5 (72.2)	48.3 (56.2)
M97	12.7	1	75/ 150	20480 *	12.5 (16.4)	25.7 (31.9)	34.5 (39.2)	45.1 (53.6)	67.8 (75.9)	60.7 (70.8)
M164	17.1	3B	50/ 300	5120	11.0 (14.5)	14.4 (17.9)	24.7 (28.1)	74.8 (88.9)	82.9 (92.8)	84.5 (98.5)

Table 4.3. Clinical, serological and agrin-induced AChR cluster results summary. * = The IgG end-titre of M97 was interpreted as an outlier.

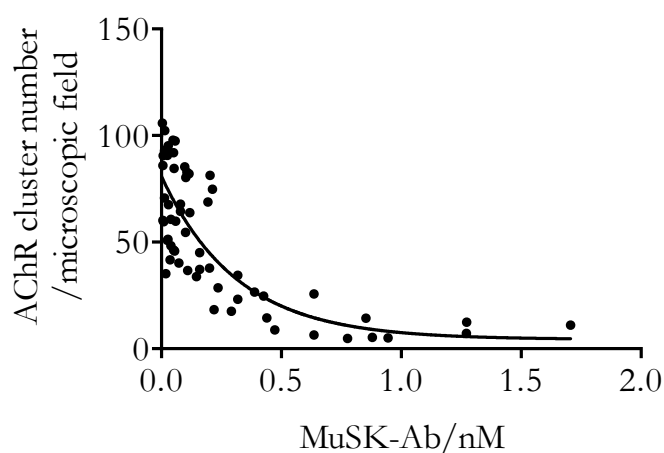


Figure 4.10. Relationship between AChR cluster number and MuSK-Ab titre/nM from 10 MuSK-MG sera

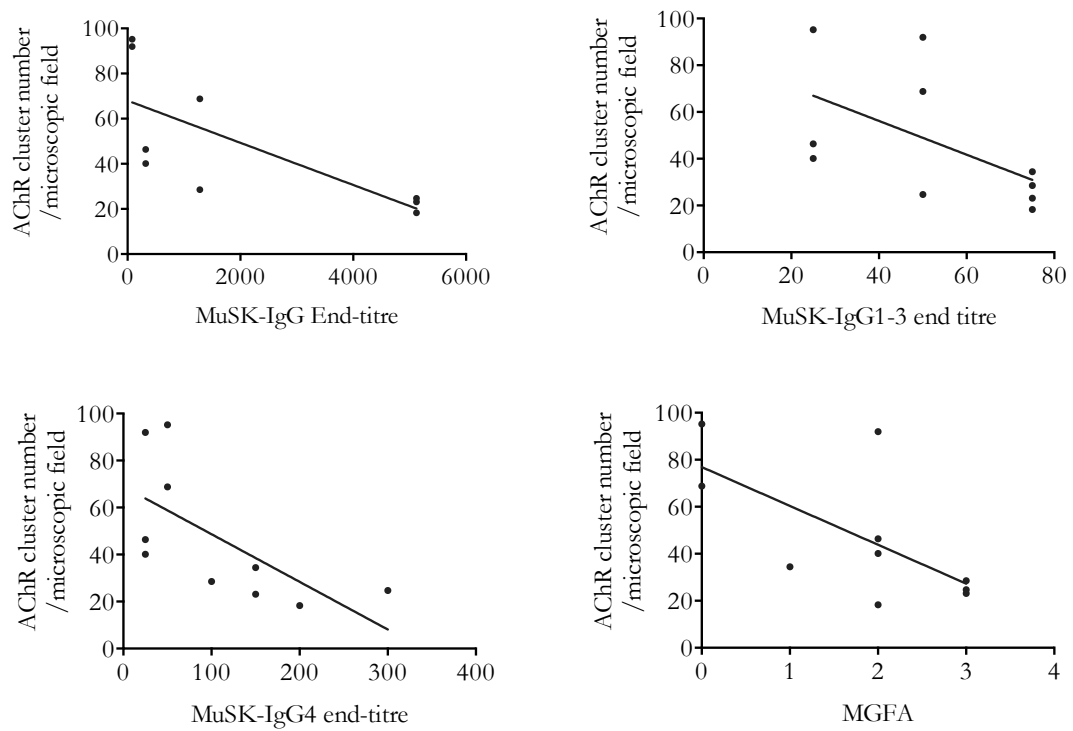


Figure 4.11. Correlation between AChR cluster number with MuSK-MG sera (1:40 dilution) and MuSK-IgG ($p=0.03$; $r^2=0.53$), MuSK-IgG1-3 ($p=0.10$; $r^2=0.31$), MuSK-IgG4 ($p=0.04$; $r^2=0.43$), and MGFA ($p=0.04$; $r^2=0.45$); (Pearson's test).

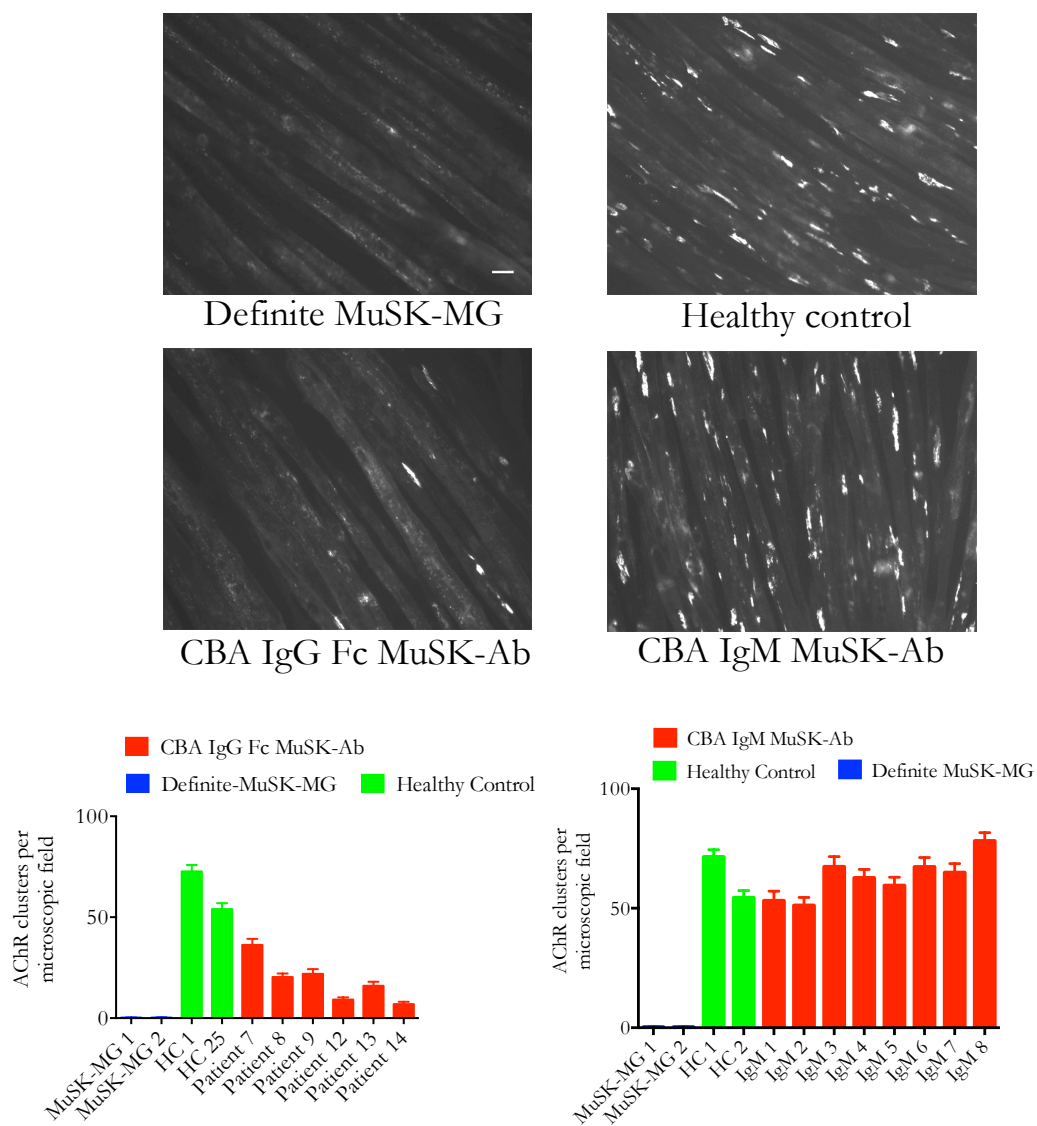


Figure 4.12. Functional effects of MuSK-IgG and -IgM on agrin-LRP4-MuSK-DOK7 clustering pathway- *Upper:* Representative images of myotubes, fluorescent labelled for AChR clusters in the presence of different sera. MuSK-MG control sera =2 and 6 MuSK-IgG CBA positive sera reduced the number of AChR clusters, but 8 CBA MuSK-IgM sera did not reduce clusters. Magnification 10x; Scale bar =50 μ m *Lower left:* Mean results from two experiments of MuSK-IgG CBA positive and control sera on AChR clusters. *Lower right:* Mean results of two experiments with MuSK-IgM CBA positive and control sera.

4.8 Discussion

The clinical features of MuSK-MG patients were similar to those reported in other Italian and North American cohorts (Evoli et al. 2003; Gup till et al. 2011).

Most patients were young females and an MGFA>2 was noted in 73% of patients at onset. Faciopharyngeal and non-MuSK-MG ‘like’ presentations were most common, with predominant neck extensor and respiratory muscle weakness presenting in older age groups. AChEIs were, as expected, poorly tolerated but remission rates were high (38%). MuSK-IgG4 was the predominant IgG subclass and correlated with disease severity. However, MuSK-IgG1-3 was also frequently detected and in a proportion of cases titres were higher than MuSK-IgG4. Lower MuSK-IgG4 and MuSK-Ab titres were associated with a higher rate of disease remission.

MuSK-IgG CBA positive patients had milder disease than MuSK-MG sera and all patients treated with immunotherapy improved. Interestingly a beneficial response to AChEIs was noted in most patients. The prognosis was favourable and not dissimilar to MuSK-MG patients. Importantly, MuSK-IgG CBA positive samples but not those containing IgM inhibited AChR clusters in cultured myotubes, confirming that pathogenic potential of Abs detected by the optimised MuSK CBA.

Three major phenotypes of MuSK-MG have been described- faciopharyngeal, non-MuSK-MG ‘like’, and predominant neck extensor and respiratory muscle weakness (Gup till & Sanders 2010). However, the explanation for these different phenotypes is unclear. The majority of MuSK-Abs bind to the first IgG like domain, and this epitope is temporally conserved, therefore idio type heterogeneity and determinant spreading of the antigen are unlikely explanations (Huijbers et al. 2016). MuSK-IgG4 was the predominant IgG subclass in MuSK-MG sera as has previously been reported (McConville et al. 2004; Ohta et al. 2007) and there were no differences in the relative proportions of other IgG subclasses between different phenotypes. MuSK protein expression levels are higher in mouse masseter

compared to gastrocnemius muscles and this may increase the likelihood of this muscle being targeted by MuSK-Abs (Viegas et al. 2012). Interestingly there was a higher proportion of patients with neck extensor and respiratory muscle weakness in older age groups and it may be that these muscles become more susceptible with advancing age.

Most patients were treated with standard therapies including steroids +/- azathioprine and rescue therapies (intravenous immunoglobulins or plasma exchange). The response to acetylcholinesterase inhibitors was poor as has previously been noted (Lavrnic et al. 2005; Evoli et al. 2008). Thymectomy was not beneficial in the 6 patients who underwent the procedure but surprisingly half of the samples had thymic histology suggestive of hyperplasia. However, thymic samples were not processed locally and the results may not be accurate (Dr. Ricciardi- personal communication). Lymph node-type infiltrates, similar to those found in AChR-MG have been noted in 75% of MuSK-MG thymic specimens and may account for some of the variation in interpretation (Leite et al. 2005).

Remission rates were higher (38%) than reports from previous studies (27%, Evoli et al. 2003; and 14%, Gupitill et al. 2011) and were not explained by the use of more novel treatments such as Rituximab. Lower titres of MuSK-Ab/nM and MuSK-IgG4 were associated with a better prognosis and higher rates of remission. However, this may be partly explained by most serum samples being drawn after treatment, the high remission rates (38%) and association of these measures to disease severity.

Although the presence of MuSK-IgG1-3 Ab has previously been reported in MuSK-MG (McConville et al. 2004; Leite et al. 2008), 29% of sera had higher titres of MuSK-IgG1-3 than MuSK-IgG4. Correlation of MuSK-IgG4 with disease severity has previously been noted (Niks et al. 2008), but similar observations with MuSK-IgG2 have not been reported.

In vivo pathogenic effects have not been noted with passive transfer of IgG1-3 from MuSK-MG patients (Klooster et al. 2012). However the lower concentrations of MuSK-Abs in IgG1-3 as compared to IgG4 fractions used in these experiments may partly explain these findings. MuSK-IgG1-3 antibodies are likely to be of pathogenic relevance as they disperse

agrin dependent and independent AChR clusters *in vitro* (Koneczny et al. 2013). However, in AChR clustering assays with serial dilutions of MuSK-MG sera, MuSK-IgG4 but not MuSK-IgG1-3 end-point titres correlated with AChR cluster number. The sample size tested was small and likely accounted for this finding. Indeed in Chapter 5, a similar analysis of a larger sample size is presented and significant correlation of all IgG subclasses was noted. Further studies to assess the mechanism of AChR cluster dispersal with IgG1-3 from MuSK-MG sera as well as passive transfer experiments in mice would be of interest. Interestingly correlation was observed between AChR cluster number and MGFA, and provides support for the use of this *in vitro* system for the assessment of novel therapeutics that will be discussed in Chapter 5.

MuSK-IgG Abs were detected in 6% of MG patients by cell-based assays. After a median follow-up of 2 years, within which risk of secondary generalisation is highest (Bever et al. 1983), ocular MG still compromised 39% of these patients. Ocular MG in MuSK-MG is rare though there are a few reports (Bennett et al. 2006; Caress et al. 2005). Interestingly a beneficial response to AChEIs was seen in 75% of patients treated with acetylcholinesterase inhibitors. By contrast 80% of the MuSK-MG cohort responded poorly or with cholinergic side effects in keeping with previous reports (Guptill et al. 2011). Immunotherapy was effective in all treated patients and a post intervention status of minimal manifestations/remission was attained in 60% of patients, equivalent to the rate in MuSK-MG patients. MuSK-MG is often associated with a particularly severe bulbar phenotype, but it is well recognized that not all patients have severe disease and it appears that the MuSK-IgG CBA positive patients are within the mild spectrum (Evoli et al. 2003).

To assess the pathogenic potential of MuSK-IgG Abs detected by CBA, AChR clustering assays were performed in cultured myotubes. All 6 sera tested reduced agrin induced AChR clusters suggesting a functional effect on the agrin/LRP4/MuSK/DOK7 pathway. Some MG sera also contained IgM that bound to and appeared specific for MuSK by colocalisation and preadsorption experiments. However, the MuSK-IgM sera tested did

not inhibit the formation of agrin-induced clusters. MuSK-IgM antibodies were detected in 11% of the MuSK-MG patients. There were no obvious differences in disease severity of these patients to suggest that the presence of IgM conferred a more severe clinical course (MGFA 0-2). Although these findings do not exclude the possibility that MuSK-IgM may act on MuSK function *in vivo*, the lack of disease specificity argues against it.

A large multinational study reported the detection of MuSK-Abs in 13% (83/633) of seronegative MG patients using a CBA, but as mentioned previously, IgM antibodies only were present in 92% (23/25) of sera (Tsonis et al. 2015). MuSK-Abs have also been reported in SNMG cohorts from Sri Lanka (3/10; 10%) and China (3/8; 38%), but in both studies an anti-human IgG(H+L) antibody that can inadvertently detect IgM was used (Chang et al. 2014; Yang et al. 2011). The results of all these studies may therefore need to be reassessed.

The experiments performed likely could have been improved with greater quantities of sera. Experiments to determine the functional relevance of MuSK-IgG antibodies by CBA were prioritised over the determination of IgG subclass and could be performed twice. In addition many samples were obtained after immunotherapies, which limits the conclusions that can be drawn from the serological analysis.

It will be important in future studies to determine if these MuSK-IgG CBA positive sera contain mainly MuSK-IgG4 Abs that can inhibit co-immunoprecipitation of MuSK and LRP4. In addition experiments to determine the pathogenic potential of CBA detected IgG4 and IgG1-3 fractions in animal models would also be of interest.

Chapter 5: Modulation of MuSK-Ab effector mechanisms

5.1 Introduction

The predominant IgG subclass fraction in Muscle specific kinase (MuSK) myasthenia gravis is IgG4 (McConville et al. 2004; Niks et al. 2008). Unlike AChR-Abs, MuSK-IgG4 Abs are monovalent and do not internalise MuSK receptors, neither do they efficiently activate the complement cascade (Koneczny et al. 2013; Leite et al. 2010; Koneczny et al. 2016). Traditionally IgG4 Abs have been thought to be anti-inflammatory and protective, but in recent years an expanding number of IgG-mediated neurological diseases have been recognised including subsets of limbic encephalitis, chronic inflammatory demyelinating polyneuropathy and pemphigus (van der Neut Kolfshoten et al. 2007; Huijbers et al. 2015).

In vitro studies have shown that Fab fragments of MuSK-IgG4 Abs disrupt the interaction between MuSK and LRP4, a critical step in the agrin/LRP4/MuSK/DOK7 (ALMD) AChR clustering pathway (**Figure 5.1**; Koneczny et al. 2013; Huijbers et al. 2013). Agrin binds to LRP4 which enhances the interaction with MuSK resulting in MuSK phosphorylation and recruitment of DOK7. The subsequent signalling cascade is less well characterised but ultimately leads to reallocation and anchoring of proteins such as rapsyn to facilitate AChR clustering. MuSK-IgG4 from two MuSK-MG patients reduced agrin-induced MuSK phosphorylation (Huijbers et al. 2013), and Ghazanfari et al. also demonstrated reduced immunolabeling of phosphorylated Src-Y418 following injection of IgG from a MuSK-MG patient in a mouse model (Ghazanfari et al. 2014). However, both MuSK-IgG4

and MuSK-IgG1-3 Abs inhibited the formation of agrin induced AChR clusters and disperse pre-formed AChR clusters (Koneczny et al. 2013). Collectively these data suggest that the disruption of MuSK/LRP4 interaction by MuSK-Abs leads inhibition of ALMD signalling and disequilibrium between AChR clustering and dispersal, but the mechanisms are still poorly described.

Current therapeutic approaches in MuSK-MG mainly involve broad immunosuppression of cell- and humoral-mediated immune responses (**Table 1.6**). Therapies such as Rituximab target B-cells specifically but are non-selective for the subsets that produce MuSK-Abs. The distinct pathogenic effect of MuSK-Abs raises the possibility of modulating the ALMD signalling downstream of MuSK/LRP4 disruption as a novel therapeutic approach in MuSK-MG.

As shown in **Figure 1.7**, MuSK phosphorylation stimulates a regulatory negative feedback loop involving SRC homology 2 domain containing phosphotyrosine phosphatase 2 (SHP2). Germline or somatic mutations of PTPN11 that result in SHP2 gain of function are associated with Noonan's syndrome (a disorder characterised by short stature, facial dysmorphism, cardiac disease, skeletal abnormalities, and an increased risk of haematological malignancy). SHP2 is expressed in C2C12 myotubes and expression of inactive and active forms increase and decrease agrin dependent AChR clustering respectively (Zhao et al 2007).

NSC-87877 is a SHP 1/2 inhibitor that binds to the catalytic cleft of SHP2, and inhibits PTP activity. Interestingly SHP2 inhibition has previously been shown to increase both agrin dependent and independent AChR clusters in C2C12 myotubes (Zhao et al. 2007).

5.1.1 Aims

- a) Confirm the effects of MuSK-IgG subclass fractions on MuSK phosphorylation
- b) Assess if SHP2 inhibition modulates the effects of MuSK-IgG subclass fractions on MuSK phosphorylation

c) Determine if SHP2 inhibition modulates the effect of MuSK-MG sera on AChR clustering.

I am very grateful to Michelangelo Cao, who purified the IgG subclass fractions and helped with the phosphorylation experiments after I had to return to Liverpool.

5.2 SHP2 inhibition causes phosphorylation of MuSK

To determine the effect of SHP2 inhibition on MuSK phosphorylation, Agrin (1:100) and NSC-87877 (1 μ M, 10 μ M, 100 μ M) were incubated with C2C12 myotubes for 40 min. MuSK phosphorylation was determined by immunoprecipitation and immunoblotting of MuSK and MuSK phosphotyrosine. Phosphotyrosine levels were normalised to the corresponding level of immunoprecipitated MuSK, and were then expressed as proportion of agrin-induced MuSK phosphorylation. As shown in

Figure 5.2, 100 μ M of NSC-87877 increased MuSK phosphorylation compared DMEM ($p=0.04$; ANOVA) and other SHP2 (1 μ M, $p=0.007$ and 10 μ M, $p=0.048$; ANOVA) but was noticeably less effective than agrin. The difference in MuSK phosphorylation between 200 μ M to 100 μ M was not significant ($p=0.10$; ANOVA). Thus SHP2 inhibition with NSC87877 increases MuSK phosphorylation independent of agrin stimulation.

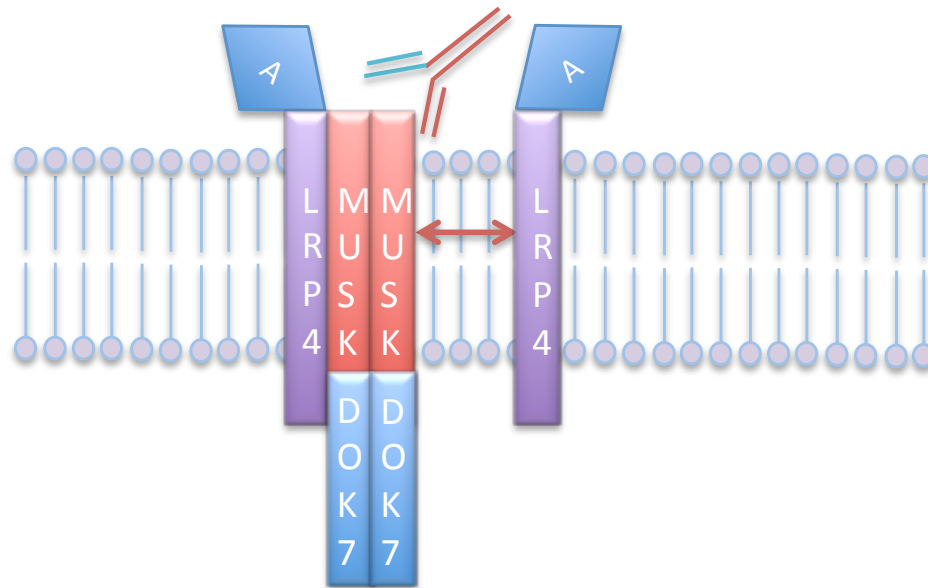


Figure 5.1. Pathogenic action of MuSK-IgG4 antibodies- Monovalent MuSK-IgG4 antibodies disrupt the interaction between MuSK and LRP4. A = Agrin; LRP4 = Low density lipoprotein receptor-related protein 4; MuSK = Muscle specific kinase; DOK7 = Docking protein 7

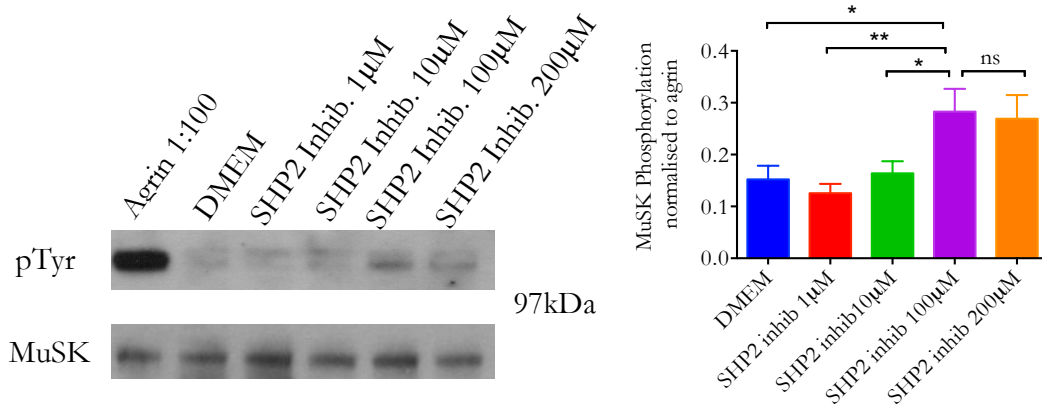


Figure 5.2. *Left:* Representative immunoblots of MuSK and phosphotyrosine. *Right:* SHP2 inhibition increases MuSK phosphorylation- C2C12 myotubes were incubated with DMEM, agrin 1:100, or SHP2 inhibitor- 1μM, 10μM, or 100μM. MuSK phosphorylation was determined by immunoprecipitation and immunoblotting of MuSK and phosphotyrosine. Phosphotyrosine levels were normalised to the corresponding level of immunoprecipitated MuSK. NSC-87877 (100μM) increased MuSK phosphorylation compared to DMEM (p=0.04; ANOVA). Data were pooled from at least 3 experiments.

5.3 MuSK-IgG4 but not MuSK-IgG1-3 antibodies reduce MuSK phosphorylation which is restored by SHP2 inhibition with NSC-87877

To determine the effect of MuSK-IgG and IgG subclass on MuSK phosphorylation, total IgG, IgG1-3, and IgG4 were purified from the plasmas of two MuSK-MG patients (Patient 6 and 12), that have been previously reported (Koneczny et al. 2013). Example elution profiles, protein concentrations and MuSK-Ab concentrations and are shown in **Figure 5.3** and **Table 5.1**.

IgG, IgG1-3 and IgG4 fractions (0.5nM) were applied to C2 myotubes with 100 μ M of NSC-87877 and/or agrin (1:800) for 40 minutes followed by immunoprecipitation and immunoblotting of MuSK and MuSK phosphotyrosine. As shown, agrin increased the level of MuSK phosphorylation compared to myotubes incubated with DMEM as expected ($p=0.0009$; ANOVA; **Figure 5.4**). When C2 myotubes were incubated with agrin and MuSK-IgG4, MuSK phosphorylation levels were lower than MuSK phosphorylation levels in samples incubated with agrin alone ($p=0.03$; ANOVA) as previously reported (Huijbers et al. 2013). By contrast, there were no differences in MuSK phosphorylation following incubation with either IgG1-3 or IgG with agrin compared to agrin alone ($p=0.79$ and $p=0.98$ respectively; ANOVA).

The addition of 100 μ M of NSC-87877 to IgG4 and agrin increased MuSK phosphorylation compared to IgG4 and agrin ($p<0.0001$; ANOVA). Normalised phosphotyrosine levels were also increased with the addition of NSC-87877 to IgG/agrin ($p=0.0008$; ANOVA) but not IgG1-3/agrin ($p=0.1$; ANOVA).

These data suggest that IgG4 Abs from MuSK-MG inhibit MuSK phosphorylation, which can be prevented by SHP2 inhibition. IgG 1-3 did not inhibit MuSK phosphorylation and nor did the total IgG, although the combined IgG1-3 and IgG4 fractions inhibited AChR clustering (Koneczny et al. 2013).

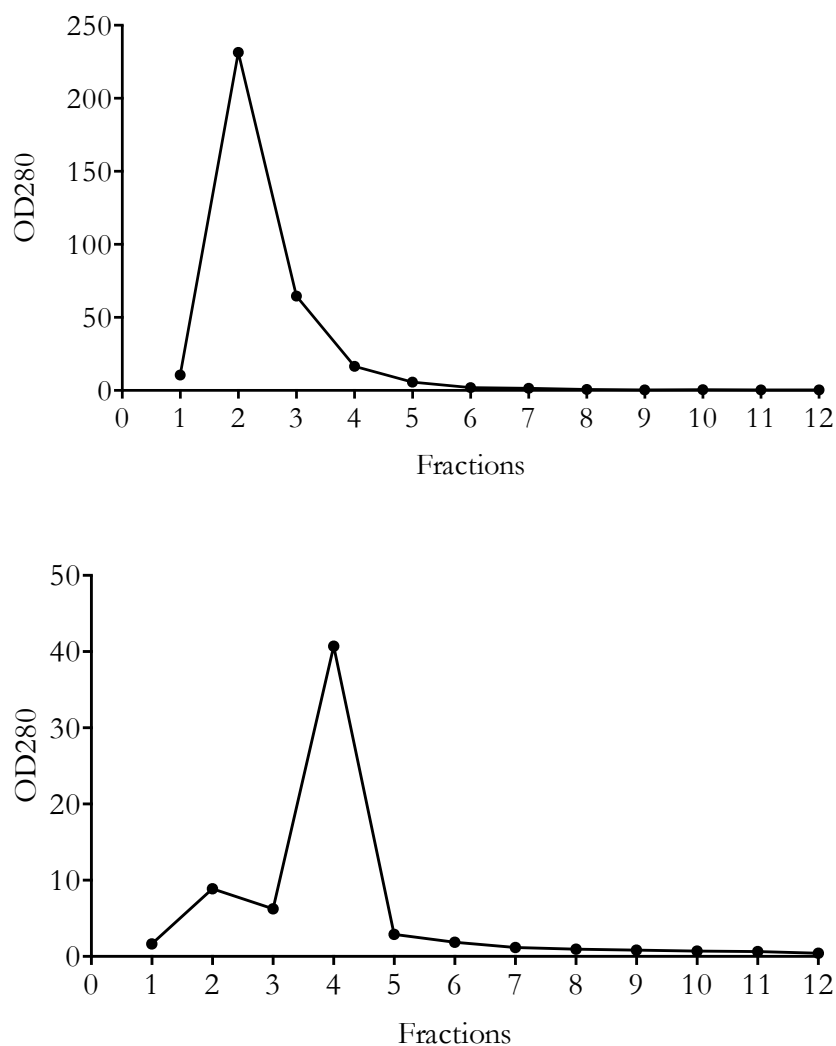


Figure 5.3. Representative elution profiles of IgG 1-3 (*top*) and IgG4 (*bottom*) from MuSK-MG plasmas

Patient	Titre purified MuSK IgG1-3 (nM)	Titre purified MuSK IgG4 (nM)	Purified IgG1-3 (mg/ml)	Purified IgG4 (mg/ml)	IgG1-3 nM/mg	IgG4 nM/mg
6	0.59	2.15	7.77	0.12	0.08	17.9
12	0.56	2.61	6.86	0.38	0.08	6.87

Table 5.1. Protein and MuSK antibody concentrations from purified IgG1-3 and IgG4 fractions.

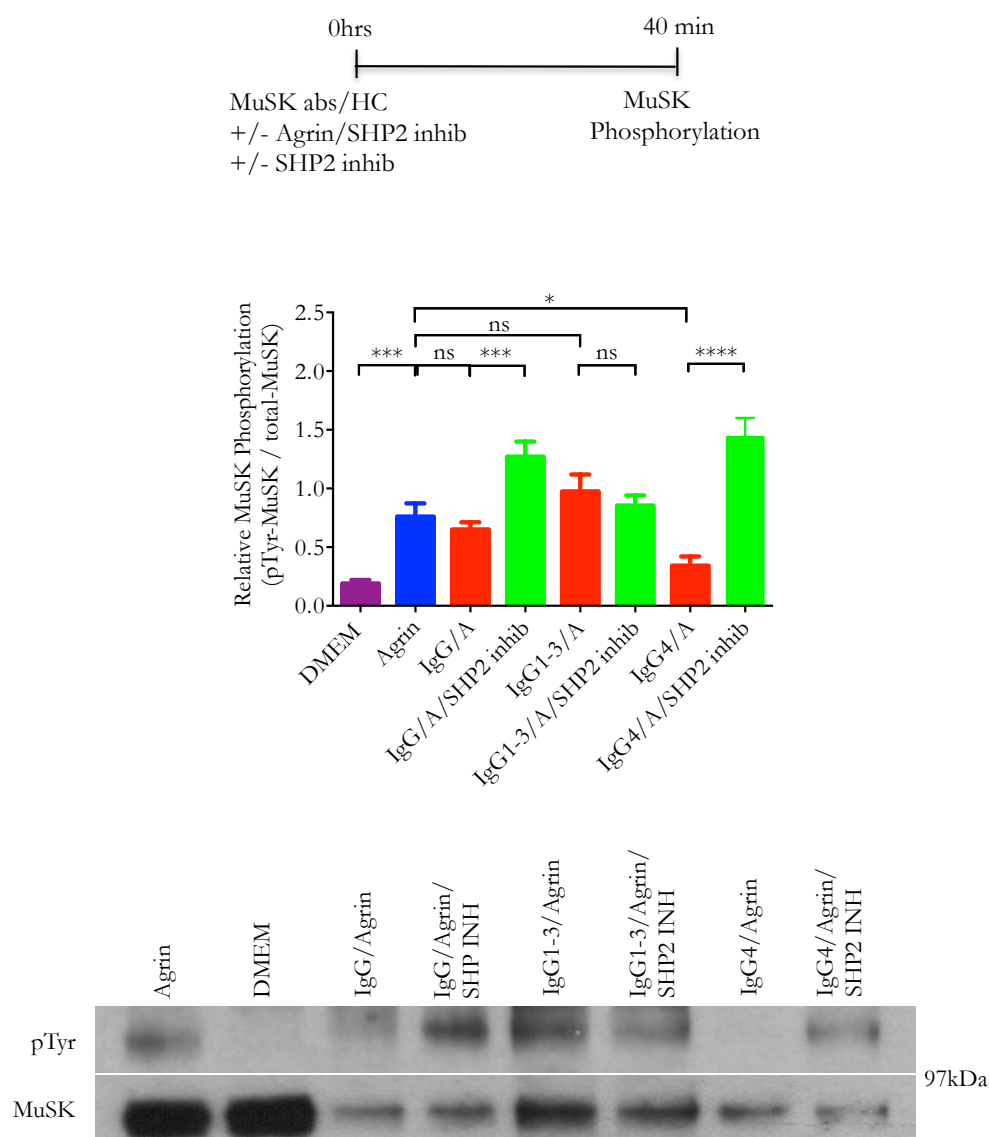


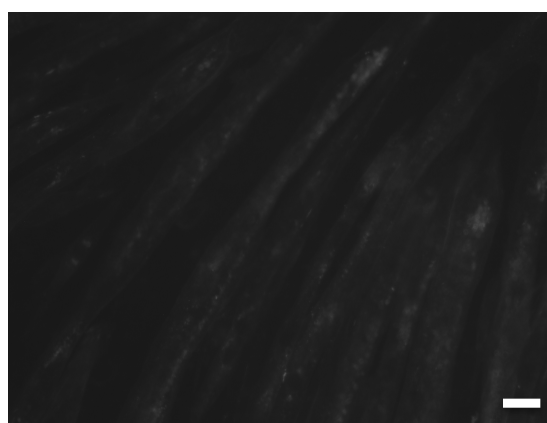
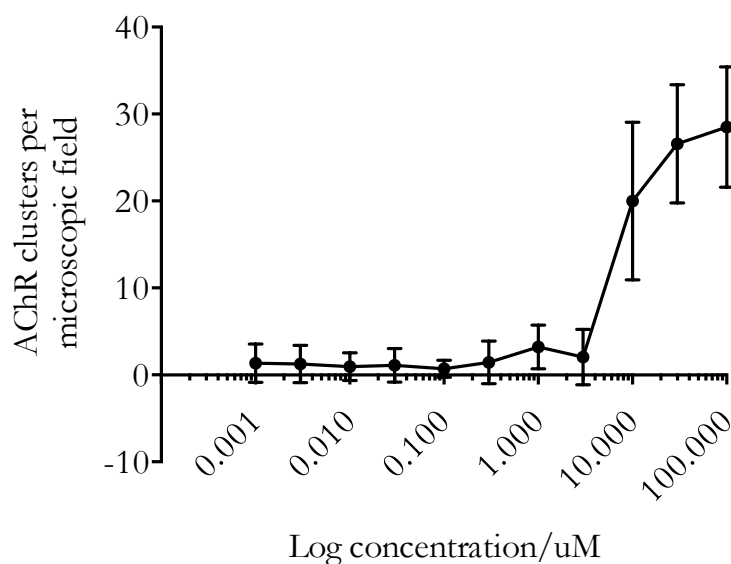
Figure 5.4. *Upper:* IgG, IgG1-3, and IgG4 subclass fractions (0.5nM) were incubated with agrin 1:800 with or without NSC-87877 100μM for 40 minutes, followed by immunoprecipitation and immunoblotting of MuSK and phosphotyrosine (MuSK was immunoprecipitated from the whole cell lysate in DMEM and agrin treated wells). *Middle:* IgG4 antibodies from MuSK-MG patients, but not IgG1-3 antibodies inhibited MuSK phosphorylation, which was restored with SHP2 inhibition (IgG4 with Agrin vs. DMEM; $p=0.03$, IgG4 with Agrin vs. IgG4, Agrin and NSC-87877; $p<0.0001$; ANOVA). *Lower:* Representative immunoblots of MuSK and phosphotyrosine

5.4 SHP2 inhibition stimulates agrin independent AChR clustering

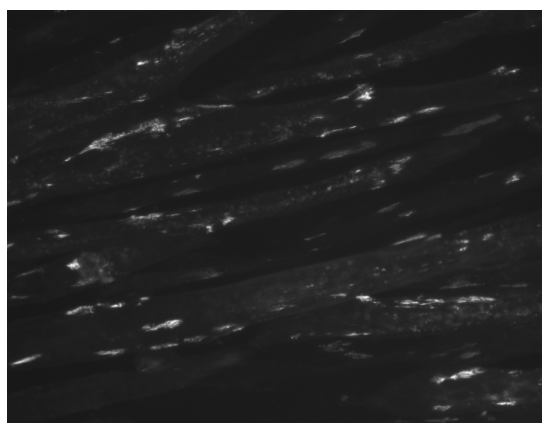
To assess the effect of SHP2 inhibition on the ALMD AChR clustering pathway, increasing concentrations of NSC-87877 were applied to C2C12 myotubes for 12 hours. AChR clusters were then stained with alpha bungaratoxin-594 (Invitrogen) and number and size were quantified. Consistent with the results observed in the MuSK phosphorylation assay, there was a dose dependent effect of NSC87877 on number and size of agrin independent AChR clusters, which appeared maximal at a concentration of 100 μ M (**Figure 5.5**).

5.5 MuSK-MG sera reduce the size and number of agrin dependent forming AChR clusters, and this effect is alleviated by SHP2 inhibition

As a preliminary experiment, the effect of SHP2 inhibition and MuSK-Abs on agrin-induced AChR clusters was assessed. Serial 1/3 dilutions of 2 MuSK-MG (10.82 and 20.72nM) and HC control sera were incubated with myotubes for 30min followed by agrin with or without NSC87877 (100 μ M) for 12 hours. To visualise AChR clusters, myotubes were labelled with fluorescent conjugated α -bungaratoxin. Images were then acquired in a blinded fashion for quantification of the number of AChR clusters. These were expressed as a proportion of the respective values of AChR clusters in myotubes incubated with HC sera. As shown in **Figure 5.6**, there was an increase in the number of AChR clusters with SHP2 inhibition in both MuSK-MG sera which was dependent on the concentration of MuSK-Abs present (MuSK-MG (10.82nM), $p=0.002-0.02$ and MuSK-MG (20.72nM), $p=0.005-0.04$; 2-way ANOVA); (pooled results from 3 experiments).



DMEM



SHP2 INH 100uM

Figure 5.5. SHP2 inhibition increases agrin independent AChR clustering- *Upper:* Different concentrations of NSC-87877 were applied to C2C12 myotubes for 12 hours followed by image acquisition and quantification of AChR clusters in a blinded fashion. Agrin independent AChR clustering appeared to saturate at a concentration of 100 μ M of NSC-87877. Error bars represent the standard deviation. *Lower:* Representative images of fluorescent-labelled AChR clusters in myotubes incubated with DMEM or NSC-87877 100 μ M. Magnification 10x; scale bar = 50 μ M

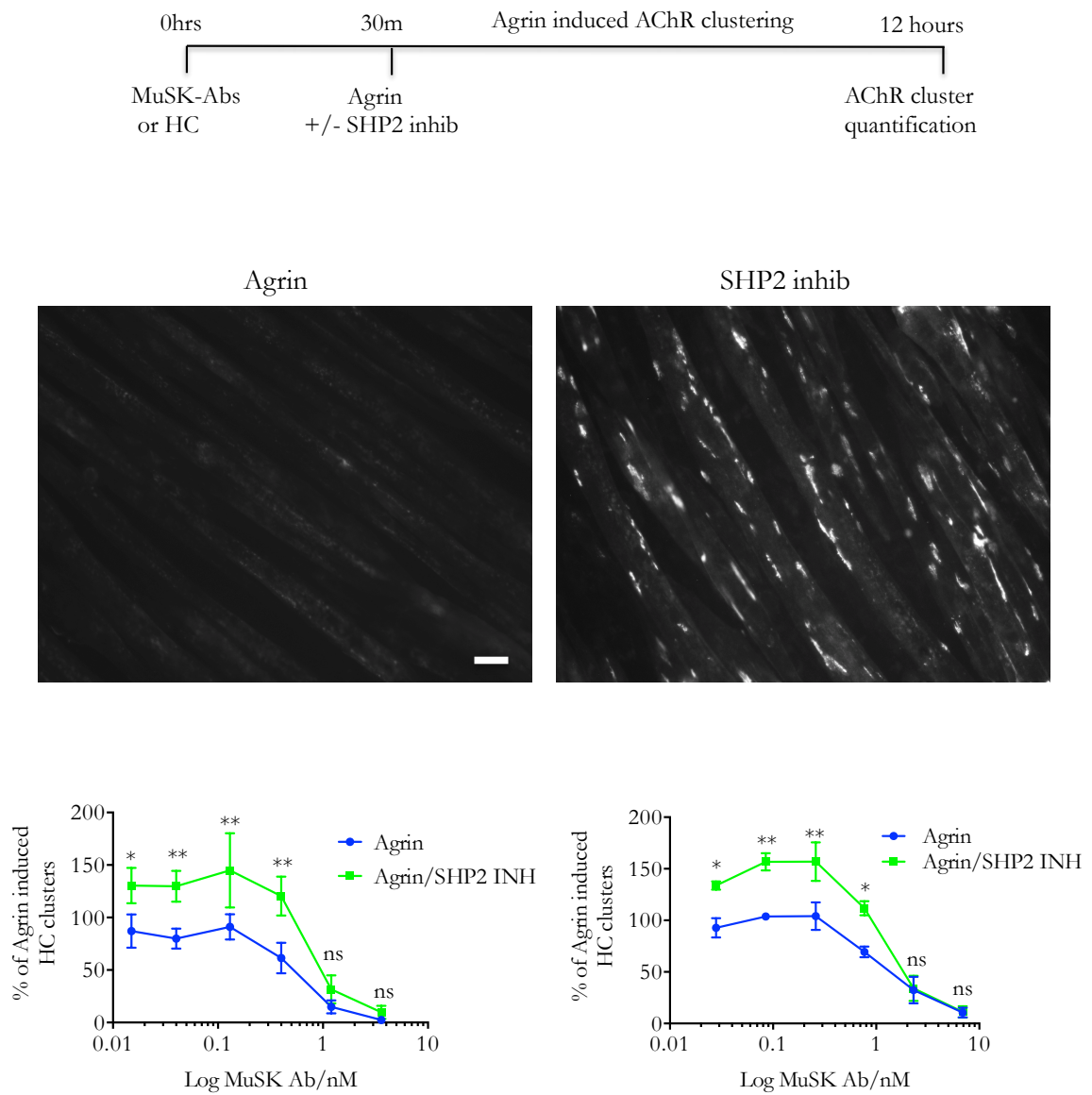


Figure 5.6. Preliminary experiments with MuSK-MG control sera and SHP2 inhibition in agrin-treated myotubes. *Upper:* MuSK-MG=2 or 2 HC sera were incubated with myotubes (30min) followed by agrin (12 hrs) after which AChR cluster quantification was performed. *Middle:* Representative images of agrin treated (*left*) and agrin/SHP2 inhibitor (*right*) myotubes. Magnification 10x, scale bar = 50 μ m *Lower left:* Data from 3 pooled experiments MuSK-MG (10.82nM) and *Lower right:* MuSK-MG (20.72nM). SHP2 inhibition resulted in an increase in the number of AChR clusters in MuSK-MG/agrin as compared to HC/agrin treated myotubes ($p=0.002-0.02$ and $p=0.005-0.04$ respectively; 2-way ANOVA).

Serum was then selected from 21 MuSK-MG patients with a range of MuSK-Ab titres (0.63-24.96nM) and clinical severities. Myotubes were incubated with MuSK-MG or HC sera followed by agrin +/- NSC-87877 for 12 hours. Based on the preliminary experiments MuSK-MG or HC sera were tested at three dilutions- 1:10, 1:30, and 1:90. In addition to the number of AChR clusters, AChR cluster area (μM) was also assessed. **Figure 5.7** (upper left) summarises the results of effect of MuSK-MG sera and SHP2 inhibition on the number of AChR clusters and representative images of myotubes are shown in **Figure 5.7** (lower). MuSK-MG sera reduced the number of AChR clusters compared to healthy controls and this effect was lessened with increased dilutions of MuSK-MG sera. MuSK-MG sera (1:10) reduced the number of AChR clusters to less than 55% of pooled HC AChR clusters, but even at the highest dilution (1:90), the quantity of AChR clusters was reduced in most myotubes incubated with MuSK-MG sera. SHP2 inhibition increased the number of AChR clusters at all three dilutions tested (1:10; $p=0.01$, 1:30; $p<0.0001$, 1:90; $p=0.0004$, Kruskal-Wallis test). At a dilution of 1:90 the number of AChR clusters was similar or greater than in respective pooled HC samples with the exception of two samples with the highest MuSK-Ab concentrations (M159 24.96 nM and M085 12.72 nM).

Figure 5.7 (middle right) summarises the effects of MuSK-MG sera on area of AChR clusters and appeared visually similar to data on the number of AChR clusters **Figure 5.7** (middle left). Indeed there was almost perfect correlation between the number and area of AChR clusters measured ($r^2=0.99$, $p<0.0001$; Pearson's test). All MuSK-MG sera (1:10) reduced the size of AChR clusters compared to pooled HC and this effect was attenuated with increasing dilution. SHP2 inhibition increased the area of AChR clusters at all three dilutions (1:10; $p=0.006$, 1:30; $p<0.0001$, 1:90; $p<0.0001$, ANOVA). Myotubes incubated with MuSK-MG sera (1:90) had AChR clusters of a similar or greater area than corresponding myotubes incubated with HC sera with the exception of M159 and M085.

Table 5.2 summarises the key clinical, serological, and experimental results.

To determine the relationship between MuSK-IgG subclass and inhibition of agrin dependent AChR clustering, correlation was assessed between IgG1-4 end-point titres and the quantity of AChR clusters. As shown in **Figure 5.8** broad correlations were observed between MuSK-IgG4 end-titres and AChR cluster number ($r^2=0.45$, $p=0.0008$), but also with MuSK-IgG1 ($r^2=0.19$, $p=0.045$), MuSK-IgG2 ($r^2=0.45$, $p=0.0009$) and MuSK-IgG3 end-titres ($r^2=0.19$, $p=0.047$); (Pearson's test). Correlation between MGFA and AChR cluster number was not significant ($r^2=-0.21$, $p=0.37$; Pearson's test).

These results demonstrate that MuSK-MG sera reduce both the number and size of forming agrin dependent AChR clusters. The effect of SHP2 inhibition is dependent on the concentration of MuSK-Abs present, particularly the IgG4 subclass fraction, but also the IgG1-3 fraction.

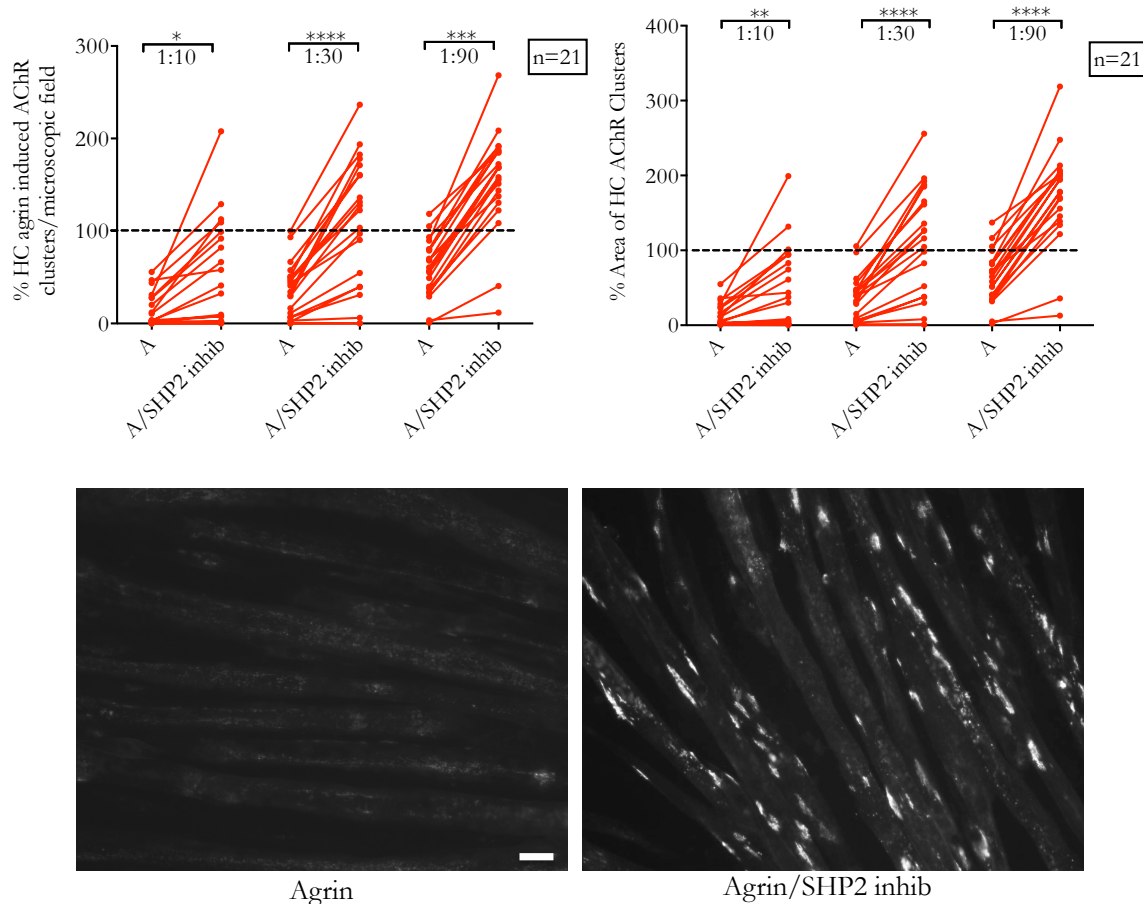


Figure 5.7. Inhibition of agrin dependent AChR cluster formation is reduced with SHP2 inhibition- Myotubes were incubated with MuSK-MG=21/HC sera=2 for 30 minutes and then agrin 1:800 with or without NSC-87877 100 μ M. After 12 hours, myotubes were fixed and stained for AChRs, and images acquired for AChR cluster quantification in a blinded fashion. *Upper left:* MuSK-MG sera inhibited the formation of agrin dependent clusters, which was dependent on the dilution of MuSK-MG sera. NSC-87877 increased the number of AChR clusters at all three dilutions tested (1:10; $p=0.01$, 1:30; $p<0.0001$, 1:90; $p=0.0004$, Kruskal-Wallis). *Upper right:* MuSK-MG sera inhibited the size of forming agrin dependent AChR clusters and this effect was alleviated with NSC-87877 (1:10; $p=0.006$, 1:30; $p<0.0001$, 1:90; $p<0.0001$; ANOVA). *Lower:* Representative images of fluorescent labelled AChR clusters in myotubes incubated with agrin or agrin/NSC-87877. Magnification 10 $\times$; scale bar = 50 μ M

Patient	MGFA sample	MUSK RIA Titer/nM	IgG1-3 Titer 1/x	IgG4 Titer 1/x	Mean AChR Cluster no (range) 1:10/1:30/1:90		AChR cluster size/ μ m 1:10/1:30/1:90		% HC cluster no 1:10/1:30/1:90		% AChR cluster size 1:10/1:30/1:90	
					A	A/S	A	A/S	A	A/S	A	A/S
M098	2b	0.25	0	0	39.0	90.4	225.4	541.5	55.7	129.1	54.8	137.2
					74.5	145.6	410.2	827.3	93.4	182.6	97.3	196.8
					86.6	134.8	485.0	710.2	118.6	184.7	137.1	200.8
M087	1	0.63	0	100	30.8	76.5	137.7	388.7	43.9	109.3	33.5	94.6
					40.2	97.7	192.2	488.7	50.4	122.5	45.6	115.9
					51.2	100.4	257.7	490.0	70.1	137.6	72.9	138.5
M105	0	0.73	75	25	32.8	40.6	148.0	178.7	46.8	58.0	36.0	43.5
					34.9	72.1	165.3	348.8	43.7	90.4	39.2	82.7
					35.9	122.9	182.8	630.7	49.1	168.4	51.7	178.3
M147	2b	1.24	25	0	19.3	64.3	95.3	341.1	27.5	91.8	23.2	83.0
					40.5	154.5	196.9	809.6	50.8	193.7	46.7	192.0
					57.6	140.1	287.9	712.9	78.9	191.9	81.4	201.5
M166	0	2.75	50	25	19.9	69.4	102.4	386.2	28.4	99.1	24.9	94.0
					53.1	128.1	262.4	679.4	66.6	160.6	62.3	161.2
					58.7	135.5	292.8	721.7	80.4	185.7	82.8	204.0
M103	0	3.42	25	25	14.2	57.5	68.4	306.0	20.2	82.0	16.6	74.4
					46.0	127.7	237.3	697.4	57.6	160.1	56.3	165.5
					49.6	136.9	249.0	731.4	68.0	187.5	70.4	206.8
M168	0	3.66	25	25	1.10	6.70	4.50	30.8	1.57	9.57	1.82	8.33
					32.8	82.5	169.7	441.6	40.8	103.5	40.3	104.8
					68.1	125.8	371.4	754.5	93.3	172.3	105	198.9
M125	2b	3.84	50	25	0.20	1.20	0.66	4.77	0.29	1.71	0.81	2.58
					5.30	24.7	23.9	119.7	6.65	30.9	6.68	29.9
					24.2	104.9	104.9	597.1	33.2	143.7	35.4	168.8
M129	1	4.15	25	100	7.45	46.6	37.1	250.6	10.6	66.5	11.3	61.0
					24.2	136.5	121.2	790.4	30.4	171.2	30.3	187.5
					58.5	152.5	298.2	876.2	80.1	208.6	84.3	247.7
M181	4b	4.37	50	150	2.50	22.7	12.7	123.6	3.57	32.4	6.19	30.1
					38.4	108.7	201.2	527.8	48.2	136.2	47.7	135.9
					41.6	123.2	206.3	690.3	57.0	168.9	58.3	195.2
M176	0	4.69	50	100	8.35	78.9	38.6	415.9	11.9	112.7	11.7	101.2
					23.4	101.7	114.4	535.2	29.3	127.5	28.6	127.0
					44.2	123.2	230.0	686.7	60.6	168.8	64.9	194.1
M175	2b	4.86	25	50	2.50	5.50	11.5	21.8	3.57	7.85	3.72	5.29
					36.9	104.7	185.7	531.2	46.2	131.2	44.1	126.0
					50.2	110.4	256.9	551.3	68.7	151.2	72.6	155.9
M149	1	6.88	50	75	0.50	1.45	1.63	5.60	0.71	2.07	1.14	2.27
					2.40	4.80	9.73	23.5	3.01	6.02	3.55	7.97
					21.3	79.2	107.4	430.7	29.2	108.5	32.0	121.8
M126	3b	7.97	50	50	1.75	28.8	8.37	154.4	2.50	41.1	4.02	37.6
					27.8	142.1	137.5	780.1	34.2	178.1	32.6	185.1
					76.7	139.6	412.2	754.5	105.1	191.2	116.5	213.3
M173	3b	8.57	50	100	21.7	145.5	117.0	819.0	31.0	207.8	28.5	199.2
					79.8	188.6	444.6	1078.2	100.0	236.5	105.5	255.8
					65.4	195.9	350.7	1127.3	89.6	268.4	99.2	318.7
M110	2b	9.25	50	50	0.95	0.80	3.28	3.16	1.36	1.14	1.59	1.4
					8.95	43.6	40.0	219.4	11.2	54.7	9.48	52.1
					29.2	141.3	141.3	602.9	40.0	155.5	40.0	170.4
M089	3b	10.1	75	150	0.40	1.40	1.35	5.54	0.57	2.00	0.94	1.69
					13.1	77.5	63.6	414.2	16.4	97.2	15.1	98.3
					40.5	115.5	211.7	627.3	55.4	158.2	51.5	177.3
M081	1	10.1	50	150	1.60	5.50	7.20	21.2	2.29	7.85	2.24	6.05
					5.50	31.4	21.2	158.5	6.90	39.4	5.91	37.6
					27.4	89.3	145.0	474.1	37.6	122.3	41.0	134.0

M085	3b	12.7	100	200	0.05	0.00	0.13	0.00	0.07	0.00	0.65	0.00
					0.10	0.15	0.36	0.49	0.13	0.19	0.87	0.77
					2.50	8.55	10.9	40.7	3.43	11.7	5.14	12.8
M097	1	12.7	75	150	1.75	1.55	6.67	6.51	2.50	2.21	2.70	2.11
					1.40	31.7	7.32	160.8	1.76	39.7	3.48	38.1
					24.6	95.3	120.2	515.3	33.6	130.6	34.0	145.7
M159	4b	25.0	175	300	0.05	0.25	0.21	1.02	0.07	0.36	1.02	0.99
					0.20	0.25	0.60	0.89	0.25	0.31	0.71	1.06
					0.55	29.6	2.21	126.7	0.75	40.6	2.09	35.8

Table 5.2. Summary of SHP2 inhibition and agrin dependent AChR cluster formation data in 21 MuSK-MG sera. A = Agrin; S = SHP2 inhibition

5.6 MuSK-MG sera disperse agrin dependent AChR clusters, and this effect is alleviated by SHP2 inhibition

To assess if MuSK-Abs disperse pre-formed AChR clusters we first assessed the stability of agrin dependent AChR clusters following agrin incubation for 12 hours. A decline in the number of AChR clusters was noted following agrin withdrawal after 12 hours. Visual scores of AChR clusters remained relatively stable up to 6 hours, but by 12 hours AChR clusters were no longer visible (**Figure 5.9**).

C2C12 myotubes were then incubated with agrin for 12 hours followed by incubation of MuSK-MG control sera (n=4, 2.87-20.72 nM) and HC sera (n=2) for 2, 3, or 6 hours. As shown in **Figure 5.10**, after 3 hours, compared to pooled HC there was a reduction in the number of AChR clusters in myotubes incubated with the two sera with the highest concentrations of MuSK-Abs (10.82 and 20.72 nM). After 6 hours this effect was also seen with the two samples of lower MuSK-Ab concentration (2.87 and 5.2nM). These experiments confirmed that agrin dependent AChR clusters were relatively stable for 6 hours, and dispersal of these AChR clusters by MuSK-MG sera could be detected within this time frame.

MuSK-MG (n=13) or HC (n=2) +/- NSC87877 were subsequently incubated with pre-formed agrin dependent AChR clusters for 6 hours (**Figure 5.11**; upper). Serum was selected from patients with MuSK-Ab titres (0.41-24.96nM). MuSK-MG sera variably reduced the number of AChR clusters compared to respective pooled HC samples at 1:10, 1:30 and 1:90 (**Figure 5.11**; lower). SHP2 inhibition attenuated this effect at all three dilutions tested (1:10; p=0.04, 1:30; p=0.0005, 1:90; p<0.0001; ANOVA). However, closer analysis of the individual data points revealed that SHP2 inhibition did not reverse completely the dispersal in sera (1:90) with highest MuSK-Ab concentrations (9.25-24.96nM).

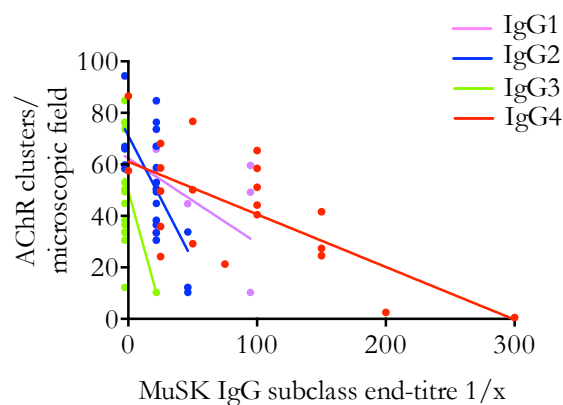


Figure 5.8. Correlation between MuSK-IgG1 ($p=0.045$; $r^2=0.19$), IgG2 ($p=0.0009$; $r^2=0.45$), IgG3 ($p=0.047$; $r^2=0.19$) and IgG4 ($p=0.0008$; $r^2=0.45$); (Pearson's test) end-titres with the number of AChR clusters was observed.

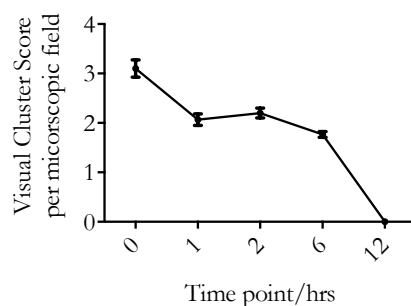


Figure 5.9. AChR cluster stability- Visual scores of AChR clusters remained relatively stabled up until the 6 hour time point

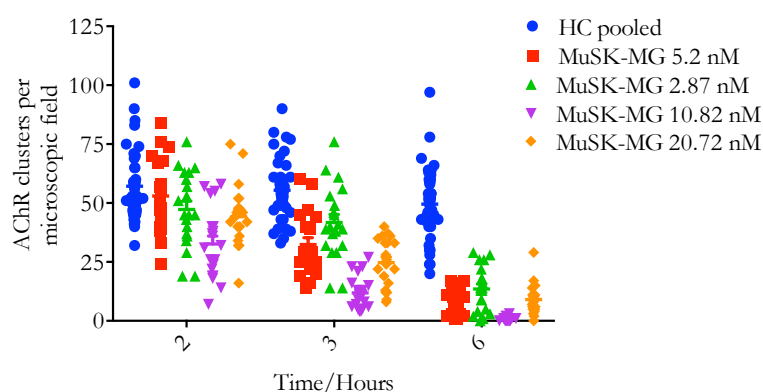


Figure 5.10. Dispersal of pre-formed agrin dependent AChR clusters- Dispersal of pre-formed AChR clusters by MuSK-MG control sera was noted at 3 hours in two samples with the highest MuSK-Ab titres (10.82 and 20.72nM) and by 6 hours in the remaining samples of lower MuSK-Ab titre (2.87 and 5.2nM)

MuSK-MG sera also reduced the area of AChR clusters and this effect was less marked with SHP2 inhibition at dilutions of 1:30 and 1:90, but not 1:10 (1:10; $p=0.06$, 1:30; 0.0008, 1:90; $p<0.0001$; ANOVA). Interestingly IgG1 ($r^2=0.48$, $p=0.009$) and IgG2 ($r^2=0.59$, $p=0.002$) end-titres correlated more strongly with AChR cluster number than MuSK-IgG4 ($r^2=0.38$, $p=0.03$); (Pearson's test). There was also weak correlation between the MGFA grade and mean AChR cluster number ($r^2=0.38$, $p=0.03$; Pearson's test).

These data suggest that MuSK-MG sera disperse agrin dependent AChR clusters, and SHP2 inhibition alleviates these effects. The MuSK-IgG1 and IgG2 subclass fractions appear to play an important role in the dispersal of agrin dependent AChR clusters in addition to the established role of the MuSK-IgG4 subclass fraction.

5.7 MuSK-MG sera disperse agrin independent AChR clusters, and this effect is alleviated by SHP2 inhibition

To assess if MuSK-Abs disperse agrin independent AChR clusters MuSK-MG sera were incubated with DOK7 overexpressing C2C12 myotubes. These myotubes exhibit high levels of basal MuSK phosphorylation and spontaneously form AChR clusters in the absence of agrin (Beeson et al. 2006).

The methodology for agrin-independent dispersal by MuSK-MG samples had been previously optimised with purified IgG subclass fractions (Koneczny et al. 2013). A preliminary experiment was performed to determine if similar results were attained with MuSK-MG control sera. DOK7 overexpressing myotubes were incubated for 13 hours with MuSK-MG control ($n=4$; 2.87-20.82 nM) and HC sera ($n=2$; **Figure 5.13**; upper). At a dilution of 1:30, the two MuSK-MG control sera with the highest MuSK-Ab concentration (10.82 and 20.72 nM) reduced the number of DOK7 AChR clusters, and at a 1:10 dilution this effect was also seen with the two MuSK-MG control sera of lower MuSK-Ab concentration (2.87 and 5.2nM); (**Figure 5.12**; middle and lower).

Patient	MGFA sample	MuSK RIA T _{titre} /nM	IgG1-3 T _{titre} 1/x	IgG4 T _{titre} 1/x	Mean AChR Cluster no (range) 1:10/1:30/1:90		AChR cluster size/ μ m 1:10/1:30/1:90		% HC cluster no 1:10/1:30/1:90		% AChR cluster size 1:10/1:30/1:90	
					A	A/S	A	A/S	A	A/S	A	A/S
M092	1	0.41	0	25	61.1	98.1	368.4	583.7	77.3	124.2	66.0	104.6
					76.7	110.3	463.5	666.0	104.9	150.8	97.8	140.5
					64.2	99.1	387.4	607.6	91.9	141.9	95.3	149.4
M166	0	2.75	50	25	25.3	61.3	125.8	344.6	33.0	77.6	22.5	61.7
					37.2	66.5	181.6	341.6	50.9	91.0	38.3	72.0
					30.9	75.8	167.2	417.8	44.3	108.5	41.1	102.7
M103	0	3.42	25	25	44.5	51.3	241.2	291.7	56.4	65.0	43.2	52.3
					31.9	80.0	153.4	454.5	43.6	108.7	32.4	95.9
					52.0	84.1	306.5	488.2	74.5	120.4	75.4	120.1
M125	2b	3.84	50	25	15.3	19.7	76.9	99.1	19.4	24.9	13.8	17.8
					38.1	63.8	195.8	350.4	52.1	87.2	41.3	73.9
					36.2	71.5	190.5	375.6	51.8	102.4	46.8	92.4
M176	0	4.69	50	100	43.1	80.4	235.4	458.7	54.6	101.8	42.2	82.2
					48.5	101.4	272.9	581.6	66.3	138.7	57.6	122.7
					44.1	98.9	229.6	559.1	63.1	141.6	56.5	137.5
M175	2b	4.86	25	50	12.8	14.4	63.8	65.9	16.2	18.2	11.5	11.8
					31.3	60.0	168.2	328.4	42.8	81.9	35.5	69.3
					31.0	76.4	180.1	420.1	45.7	109.3	44.3	103.3
M154	1	6.88	25	50	21.9	23.2	113.5	119.4	2.7	29.4	20.3	21.4
					29.3	76.8	156.2	438.8	40.0	105.1	32.9	92.6
					34.4	71.9	193.2	420.3	49.2	102.9	47.5	103.4
M173	3b	8.57	50	100	35.9	45.8	191.6	216.5	44.5	57.9	34.3	38.8
					48.4	101.7	273.2	606.2	66.1	139.1	57.6	127.9
					45.6	76.4	268.2	559.2	65.3	139.2	66.0	137.5
M110	2b	9.25	50	50	12.0	15.2	241.2	291.7	15.2	19.2	10.1	14.2
					25.2	48.3	153.4	454.5	34.4	66.1	25.0	58.0
					30.8	53.1	306.5	488.2	44.1	76.0	41.2	71.6
M089	3b	10.1	75	150	7.00	8.95	31.9	37.0	8.86	11.3	5.72	6.62
					12.4	27.2	52.9	128.0	17.0	37.1	11.2	27.0
					19.2	40.3	92.9	205.9	27.4	57.6	22.8	50.6
M081	1	10.1	50	150	17.0	19.9	70.8	89.3	21.5	25.2	12.7	16.0
					23.2	25.8	111.5	122.3	31.7	35.2	23.5	25.8
					42.7	61.5	242.3	334.0	61.2	88.0	59.6	82.1
M085	3b	12.7	100	200	7.00	10.8	31.3	46.7	8.86	13.7	4.66	8.37
					8.75	13.6	37.1	60.5	12.0	18.5	10.9	12.8
					12.2	17.5	58.0	86.5	17.4	25.0	14.3	21.3
M159	4b	25.0	175	300	18.8	23.4	89.0	115.9	23.7	29.6	16.0	20.8
					19.5	20.3	97.9	103.6	26.7	27.8	20.5	21.9
					17.7	33.7	92.4	164.6	25.3	48.2	22.7	40.5

Table 5.3. Summary of SHP2 inhibition and agrin dependent AChR cluster dispersal data in 13 MuSK-MG sera. A = Agrin; S = SHP2 inhibition

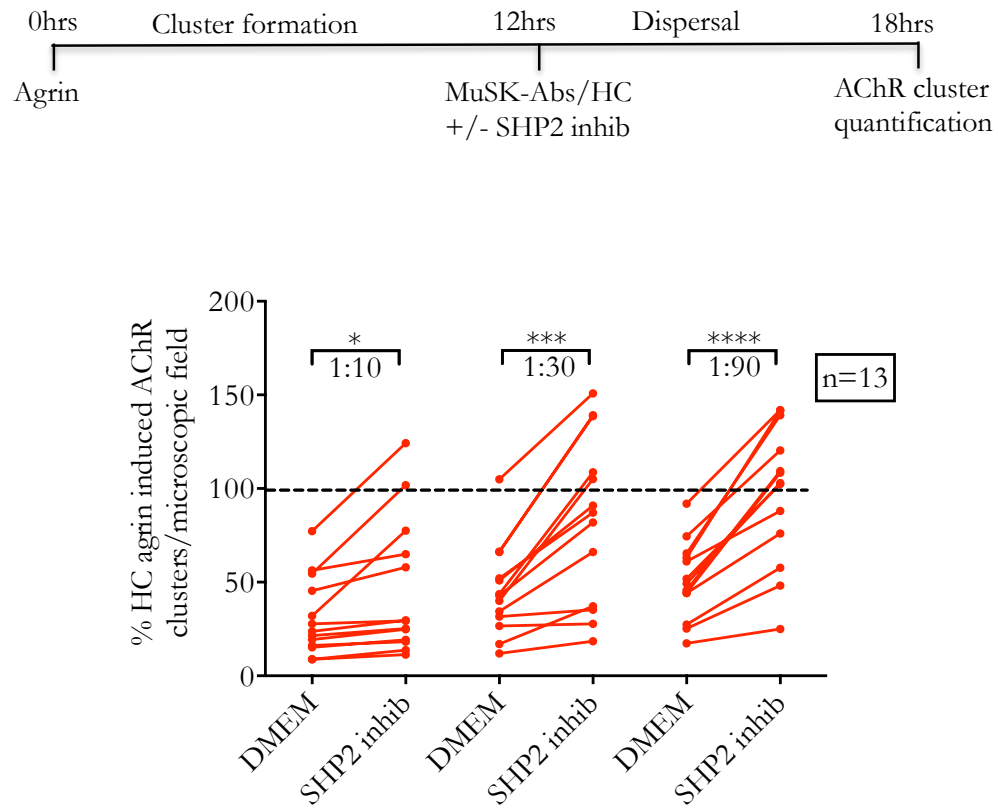
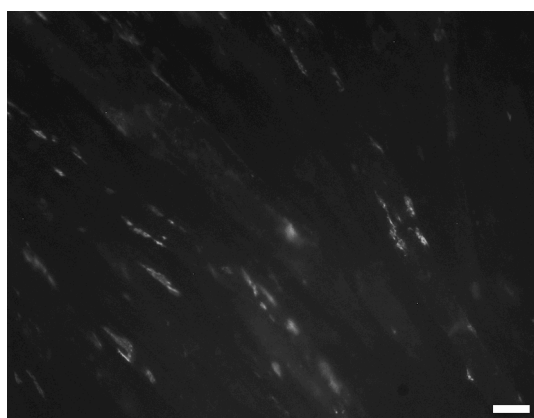
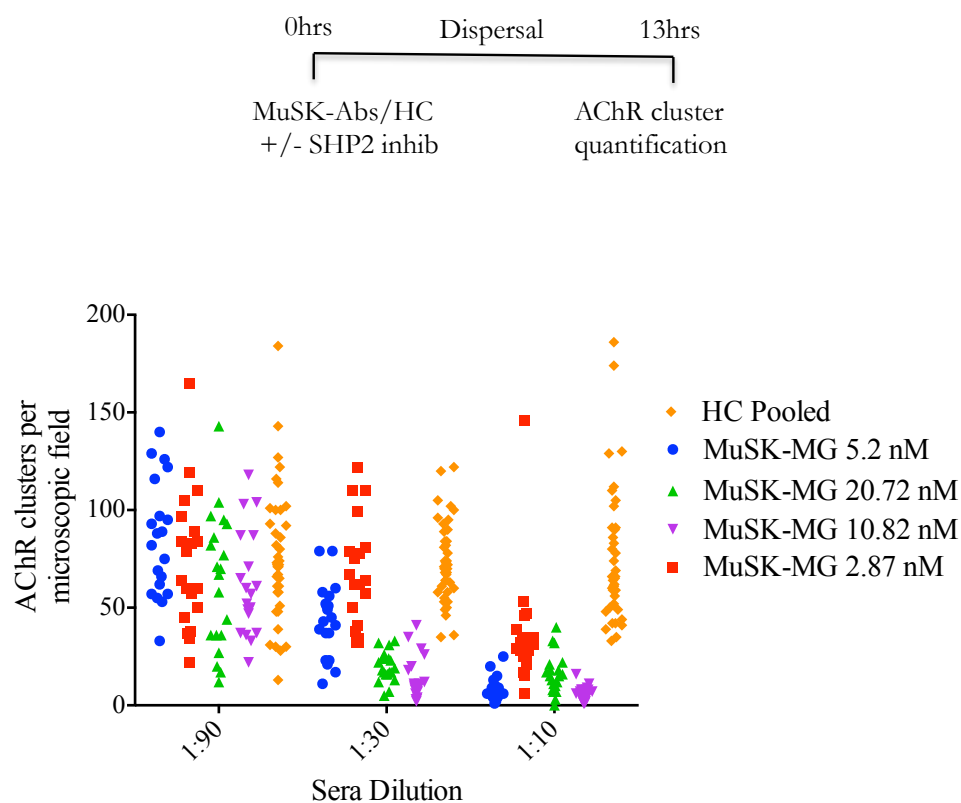
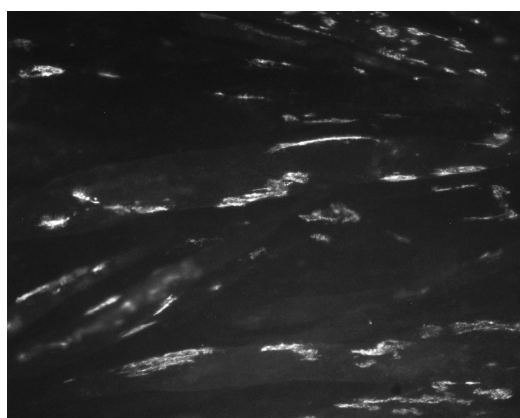


Figure 5.11. Dispersal of preformed agrin dependent AChR clusters is reduced with SHP2 inhibition- *Upper:* AChR clusters were stimulated with agrin (1:800) for 12 hours, followed by incubation with MuSK-MG sera=13/HC sera=2 with or without NSC-87877 100 μ M for 6 hours. Myotubes were then fixed and stained for AChRs, and images acquired for AChR cluster quantification in a blinded fashion. *Lower:* MuSK-MG sera reduced the number of pre-formed agrin dependent AChR clusters and this effect was alleviated by NSC-87877 (1:10; $p=0.04$, 1:30; $p=0.0005$, 1:90; $p<0.0001$; ANOVA)



DMEM



SHP2 INH

Figure 5.12. Preliminary DOK7 AChR cluster dispersal experiment. *Upper:* DOK7 overexpressing myotubes were incubated with 4 MuSK-MG and 2 HC sera for 13 hours, followed by AChR cluster quantification. *Middle:* Summary of results with 4 MuSK-MG control sera (2.87-20.72nM) and 2 HCs. *Lower:* Representative images of myotubes incubated with either DMEM or SHP2 inhibitor. Magnification 10x, scale bar = 50 μ M

The experiment was subsequently repeated with MuSK-MG sera (n=14) and HC sera (n=2). Sera were selected from patients with MuSK-Ab titres (1.24-24.96nM). As shown in **Figure 5.13**, MuSK-MG sera reduced the number of DOK7 AChR clusters. Closer analysis of the individual data points at 1:10 revealed two broad groups, of which one group (4.14-24.96nM) reduced DOK7 AChR clusters more effectively than the other (1.24-8.57nM). SHP2 inhibition reduced the dispersal of DOK7 AChR clusters all three dilutions tested (1:10; $p=0.002$, 1:30; $p<0.0001$, 1:90; $p=0.004$; ANOVA) but on visual inspection the effect was less pronounced at a dilution of 1:90.

MuSK-MG sera also reduced the area of DOK7 AChR clusters. Similar to the above results, at a 1:10 dilution there was a variable reduction in the area of DOK7 AChR clusters by the same two groups of MuSK-MG sera noted above. SHP2 inhibition alleviated the reduction in area of DOK7 AChR clusters at 1:10 and 1:30 ($p=0.004$ and $p=0.0004$ respectively; ANOVA) but not at 1:90 ($p=0.66$; ANOVA). At this dilution the majority of myotubes without NSC-87877 contained AChR clusters that were not dissimilar in size to myotubes incubated with pooled HC, but in some cases the addition of NSC-87877 appeared to adversely affect this measure. This may have been due to technical error but the effect appeared consistent, and was present in multiple samples. The addition of SHP2 inhibitor may have resulted in a saturating effect on MuSK phosphorylation (please see Discussion).

Table 5.4 summarises the key clinical, serological, and experimental results

Finally the dispersal of DOK7-AChR clusters broadly correlated with MuSK-IgG1 ($r^2=0.36$, $p=0.02$) and IgG4 ($r^2=0.5$, $p=0.005$), but not MuSK-IgG2 and IgG3 end-titres ($r^2=0.05$, $p=0.46$ and $r^2=0.19$, $p=0.12$ respectively); (Pearson's test). No correlation was observed between MGFA and number of AChR clusters ($r^2=0.10$, $p=0.27$; Pearson's test).

These results suggest that MuSK-MG sera disperse agrin independent AChR clusters, and this correlates with MuSK-IgG1 and MuSK-IgG4 end-point titres. SHP2 inhibition ameliorates these effects but maybe detrimental in the DOK7 overexpressing model depending on MuSK-Ab concentration.

Patient	MGFA sample	MuSK RIA Titer/nM	IgG1 Titer 1/x	IgG4 Titer 1/x	Mean AChR Cluster no (range) 1:10/1:30/ 1:90		AChR cluster size/ μ m 1:10/1:30/ 1:90		% HC cluster no 1:10/1:30/ 1:90		% AChR cluster size 1:10/1:30/ 1:90	
					A	A/S	A	A/S	A	A/S	A	A/S
M147	2b	1.24	25	0	70.7	97.5	395.6	501.8	84.1	116	82.2	104.3
					120.7	186.6	746.5	1099.6	127.8	197.7	132.5	195.2
					100.6	145.8	600.3	812.0	79.9	115.8	78.1	105.6
M103	0	3.42	25	25	94.0	144.9	533.9	800.0	111.8	172.4	110.9	166.2
					117.3	177.2	748.4	1016.1	124.3	187.8	132.8	180.3
					149.9	172.5	981.5	1010.2	119.1	137.0	127.6	131.4
M168	0	3.66	25	25	59.2	128.7	309.9	749.2	70.4	153.1	64.4	155.6
					130.6	173.0	860.6	1062.0	138.3	183.3	152.7	188.5
					123.5	152.1	805.3	924.0	98.2	120.8	104.7	120.2
M129	1	4.15	25	100	18.8	42.4	101.3	205.0	22.3	50.5	19.0	42.6
					71.4	117.7	411.0	640.0	75.7	124.7	73.1	113.6
					117.4	146.5	731.9	817.8	93.2	116.4	95.2	106.3
M181	4b	4.37	50	150	11.8	25.6	56.4	137.0	14.0	30.5	11.7	28.5
					46.3	91.6	276.5	506.9	49.0	97.1	49.1	90.0
					113.9	118.6	720.0	678.9	90.4	94.2	93.6	88.3
M176	0	4.69	50	100	80.4	139.0	442.9	802.8	95.6	165.4	92.0	166.8
					105.8	173.5	674.0	1009.1	112.1	183.8	119.6	179.1
					137.1	166.3	868.8	914.5	108.9	132.1	113.0	118.9
M175	2b	4.86	25	50	61.2	107.8	312.6	559.8	72.8	128.2	64.9	116.3
					141.4	157.9	870.5	903.4	149.8	167.3	154.5	160.3
					131.3	157.1	876.6	906.4	104.3	124.8	114.0	117.9
M149	1	6.88	50	75	14.9	25.3	66.4	114.1	17.7	30.1	13.8	23.7
					42.0	60.3	220.8	312.7	44.5	63.9	39.2	55.5
					98.1	111.0	540.9	587.6	77.9	88.2	70.3	76.4
M126	3b	7.97	50	50	27.0	31.1	124.2	140.6	32.1	37.0	25.8	29.2
					64.5	74.2	368.5	395.5	68.3	78.6	65.4	70.2
					108.0	102.6	677.3	516.9	85.6	81.5	88.1	67.2
M173	3b	8.57	50	100	63.8	136.8	328.5	764.8	75.9	162.7	68.2	158.9
					126.3	139.1	789.5	793.4	133.8	147.4	140.1	140.8
					150.6	159.4	1008.1	900.1	119.6	126.6	131.2	117.0
M110	2b	9.25	50	50	21.7	56.4	100.2	284.2	25.8	67.1	20.8	59.0
					83.9	186.8	529.3	1095.2	88.9	197.9	93.9	194.4
					136.6	192.9	938.8	1095.0	110.9	153.2	122.0	142.4
M089	3b	10.1	50	150	22.3	29.3	107.9	145.5	26.5	34.8	22.4	30.2
					60.5	110.2	339.2	620.4	64.1	116.8	60.2	110.1
					131.1	112.0	807.2	607.9	104.1	88.9	105.0	70.0
M081	1	10.1	50	150	34.6	19.3	161.3	85.4	41.1	23.0	33.5	17.7
					32.3	78.9	167.9	428.4	34.1	83.6	29.8	76.0
					97.7	126.2	612.0	695.3	77.6	100.2	79.6	90.4
M159	4b	25.0	175	300	22.6	52.2	107.0	280.1	26.8	62.1	22.2	58.2
					23.5	51.8	121.2	271.5	24.9	54.9	21.5	48.2
					48.1	90.0	268.6	486.3	38.3	71.5	34.9	63.2

Table 5.4. Summary of SHP2 inhibition and agrin independent (DOK7) AChR cluster dispersal data from 14 MuSK-MG sera. A = Agrin; S = SHP2 inhibition

5.8 Re-formation of agrin induced AChR clusters following dispersal by MuSK-Abs is increased with SHP2 inhibition

To determine if SHP2 inhibition increased the re-formation of agrin dependent AChR clusters following dispersal, myotubes were incubated with agrin for 12 hours, MuSK-MG sera (n=14) and HC sera (n=2)) were then added for 6 hours, and finally myotubes were re-incubated with agrin conditioned media for 12 hours (**Figure 5.14**; upper). Sera were selected from patients with MuSK-Ab titres (0.25-17.47nM).

SHP2 inhibition increased the number of AChR clusters (1:10, $p=0.002$, 1:30; $p=0.008$, 1:90; $p<0.0001$; ANOVA; **Figure 5.14**) and size of AChR clusters at 1:10 and 1:90 (1:10; $p=0.006$, 1:30; $p=0.08$, 1:90; $p<0.0001$; ANOVA). There were two samples at different dilutions (1:30 and 1:90) which, when incubated NSC-87877, resulted in fewer and smaller AChR clusters. However, this effect appeared more sporadic than was noted in DOK7 overexpressing myotubes and may have been due to technical error. There was no correlation with MGFA ($r^2=0.003$, $p=0.86$; Pearson's test) but end-point titres of IgG1 ($r^2=0.3$, $p=0.04$), IgG2 ($r^2=0.30$, $p=0.04$) and IgG4 ($r^2=0.32$, $p=0.04$); (Pearson's test) broadly correlated with the number of AChR clusters. **Table 5.5** summarises the key clinical, serological, and experimental results. These data suggest that following dispersal by MuSK-MG sera, SHP2 inhibition enhances the recovery of agrin dependent AChR clusters.

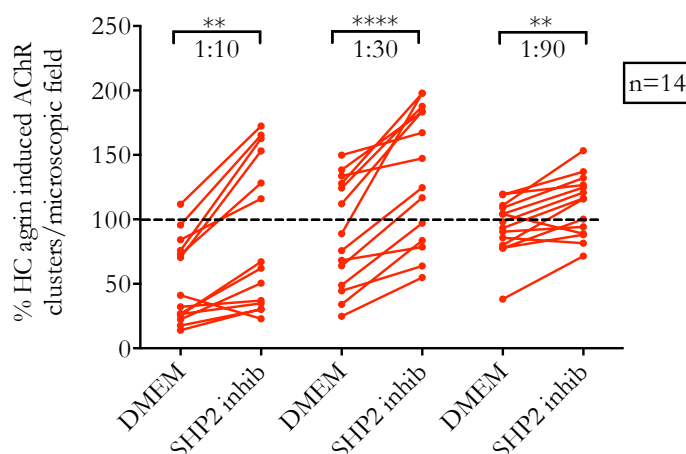


Figure 5.13. Dispersal of pre-formed agrin independent AChR clusters in DOK7 overexpressing myotubes is reduced with SHP2 inhibition- DOK7 overexpressing myotubes were incubated with MuSK-MG=14/HC sera=2 with or without NSC-87877 100 μ M for 13 hours. Myotubes were then fixed and stained for AChRs and images acquired for AChR cluster quantification in a blinded fashion. MuSK-MG sera reduced the number of AChR clusters compared to myotubes incubated with pooled HC sera. This effect was attenuated with NSC-87877 but was less marked at 1:90 (1:10; $p=0.002$, 1:30; $p<0.0001$, 1:90; $p=0.004$; ANOVA).

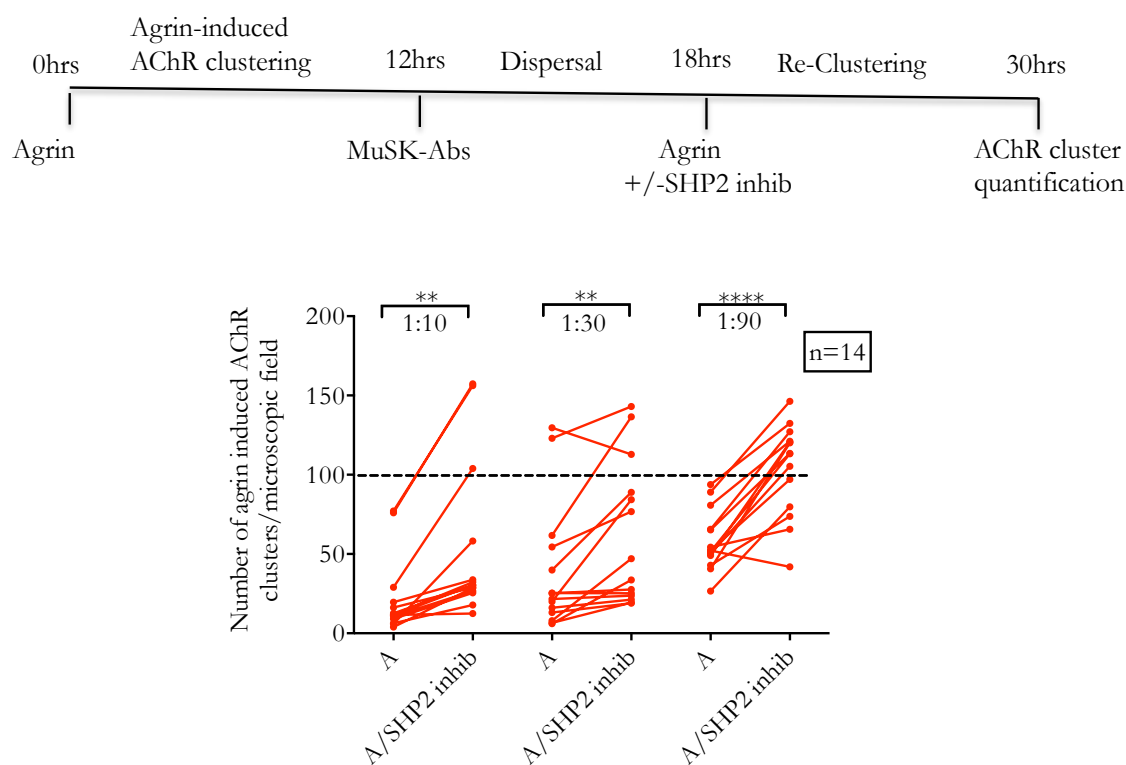


Figure 5.14. Following dispersal of agrin dependent AChR cluster, reformation of AChR cluster is increased with SHP2 inhibition- *Upper:* Myotubes were incubated with agrin for 12 hours, then MuSK-MG=14/HC sera=2 for 6 hours, followed by re-incubation with agrin for a further 12 hours. Myotubes were then fixed and stained for AChRs and images acquired for AChR cluster quantification in a blinded fashion. *Lower:* SHP2 inhibition increased the recovery of AChR clusters (1:10, $p=0.002$, 1:30; $p=0.008$, 1:90; $p<0.0001$; ANOVA).

Patient	MGFA sample	MuSK RIA Titer/ μ M	IgG1 Titer 1/x	IgG4 Titer 1/x	Mean AChR Cluster no (range) 1:10/1:30/1:90		AChR cluster size/ μ m 1:10/1:30/1:90		% HC cluster no 1:10/1:30/1:90		% AChR cluster size 1:10/1:30/1:90	
					A	A/S	A	A/S	A	A/S	A	A/S
M098	2b	0.25	0	0	41.4	85.6	209.4	408.3	76.1	157.3	50.9	99.3
					79.3	92.2	444.2	470.2	123.0	143.0	105.4	111.5
					59.2	97.2	332.2	513.3	89.1	146.3	93.9	125.1
M092	1	0.41	0	25	42.0	85.0	219.0	420.2	77.2	156.2	53.3	102.2
					83.6	72.8	468.8	379.8	129.6	112.8	112.2	90.1
					43.3	75.4	237.7	408.6	65.2	113.5	67.2	115.5
M125	2b	3.84	50	25	6.15	16.6	25.0	62.6	11.3	30.4	6.75	15.2
					25.8	57.3	125.2	282.6	39.9	88.9	29.7	67.1
					53.7	80.5	285.3	439.0	80.8	121.1	80.7	124.1
M165	2b	5.27	50	200	15.8	56.5	69.8	243.6	29.0	103.9	17.0	59.3
					35.1	49.5	181.0	255.0	54.4	76.8	42.9	58.8
					27.1	79.8	143.4	441.5	40.7	120.1	40.5	114.4
M133	0	6.54	50	100	3.25	31.7	11.6	158.3	5.98	58.2	3.51	38.5
					39.8	88.1	223.6	475.7	61.7	126.6	53.1	112.9
					62.3	88.0	365.6	503.3	93.8	132.4	103.4	142.3
M083	2b	6.88	50	200	5.80	14.5	21.9	54.5	10.7	26.7	5.91	13.2
					5.15	30.4	19.6	135.8	7.99	47.1	4.91	32.2
					32.6	80.1	172.1	399.3	49.0	120.5	48.6	112.9
M154	1	6.88	25	50	6.35	17.6	24.8	70.7	11.7	32.3	6.03	7.2
					12.9	54.4	61.1	255.0	19.9	84.3	12.5	60.5
					43.6	84.5	246.4	441.5	65.6	127.1	69.7	124.8
M167	3b	9.45	75	100	2.15	15.6	8.50	64.0	3.95	28.6	3.45	15.5
					3.90	21.7	14.1	98.1	6.05	33.7	3.73	23.3
					34.7	54.5	192.9	342.4	52.2	97.0	54.5	96.8
M116	0	9.65	100	50	6.30	6.75	24.9	24.7	11.6	12.4	6.06	6.01
					4.25	12.5	16.2	47.8	6.59	19.3	4.52	11.3
					33.3	670.0	176.7	349.6	50.1	105.3	50.0	98.8
M081	1	10.1	50	150	3.45	9.7	12.6	36.2	6.34	17.8	3.41	8.81
					8.45	12.3	34.6	46.8	13.1	19.0	8.65	11.1
					36.1	43.6	189.5	219.4	54.2	65.6	53.6	62.0
M093	3b	12.7	125	100	6.85	16.5	25.1	62.9	12.6	30.3	6.41	15.3
					16.3	17.8	67.6	69.5	25.3	27.6	16.0	16.5
					28.5	49.0	152.9	242.9	42.9	73.7	43.2	68.7
M180	2b	12.7	125	100	10.7	18.4	42.2	71.0	19.6	33.8	10.3	17.3
					16.4	16.3	69.0	62.0	25.4	25.3	16.4	14.7
					34.7	27.9	181.3	129.6	52.2	41.9	51.2	36.6
M097	1	12.7	75	150	8.85	15.7	33.1	64.4	16.3	28.8	8.94	16.5
					14.0	15.4	63.2	63.0	21.6	23.8	15.8	15.0
					34.3	75.2	183.8	386.3	51.6	113.2	52.0	109.2
M128	0	17.5	150	200	4.95	13.9	18.6	51.5	9.10	25.5	4.77	13.2
					10.4	13.7	43.9	52.0	16.1	21.3	10.4	12.3
					17.7	53.0	82.9	268.6	26.7	79.8	23.5	75.9

Table 5.5. Summary of SHP2 inhibition and re-formation of agrin dependent AChR clusters data from 14 MuSK-MG sera. A = Agrin; S = SHP2 inhibition

5.9 Discussion

These experiments demonstrate that purified IgG4 but not IgG1-3 or IgG from MuSK-MG plasmas reduce MuSK phosphorylation. Using NSC-87877, a SHP2 inhibitor, tyrosine phosphorylation of MuSK and AChR clustering were increased without agrin stimulation. The addition of NSC-87877 restored MuSK phosphorylation in myotubes incubated with MuSK-IgG4 subclass fractions. Importantly NSC-87877 attenuated the inhibition on formation of agrin dependent and dispersal of agrin dependent/independent AChR clusters by MuSK-MG sera. NSC-87877 enhanced recovery of agrin dependent AChR clusters following dispersal by MuSK-MG sera.

Downstream signalling of the agrin/LRP4/MuSK/DOK7 pathway is dependent on the formation of a 2:2 heterotetramer composed of the heparan sulphate proteoglycan agrin, its ligand LRP4, and MuSK. This structure enhances the interaction between MuSK and LRP4, facilitates MuSK dimerization, and stimulates auto- and trans- phosphorylation of the juxtamembrane region of MuSK (Zong et al. 2012; Hopf & Hoch 1998). The subsequent recruitment and dimerization of the adaptor protein DOK7 further stimulates MuSK trans-phosphorylation and in turn recruits the adaptor proteins Crk and Crk-L (Burden et al. 2013). Downstream of MuSK activation a number of signalling pathways have also been implicated including Src and Abl kinases, geranylgeranyl transferase 1 (GGT), GTPases of the Rho family, and PAK1 (which is activated by Rho GTPases). Selective agonists of these 'pro-clustering' kinases would appear a logical therapeutic strategy in MuSK-MG but might in theory increase the risk of malignancy as many of the aforementioned kinases are upregulated in solid tumours. An alternative strategy would be to selectively antagonise inhibitors of AChR clustering. MuSK phosphorylation is negatively regulated by the ubiquitously expressed cytosolic non-receptor phosphotyrosine phosphatase Shp2. Structurally SHP2 consists of two SH2 domains, a catalytic protein tyrosine phosphatase (PTP) domain, a c-terminal tail with tyrosyl phosphorylation sites (Y542 and Y580), and a

proline-rich motif. The n-terminal SH2 domain inhibits the catalytic PTP domain in the basal state, but upon stimulation undergoes a conformational change to facilitate activation of the PTP domain. NSC-87877 is a SHP1/2 inhibitor that binds to the catalytic cleft of SHP2, and inhibits protein tyrosine phosphatase (PTP) activity. Shp2 expression unlike Shp1 is readily detected C2C12 myotubes, suggesting that previously observed increases in agrin dependent and independent AChR clustering with NSC-87877 were specific to Shp2 inhibition (Madhavan et al. 2005; Chen 2006; Zhao et al 2007).

To assess the therapeutic potential of SHP2 inhibition in MuSK-MG, the effects of IgG subclass fractions on MuSK phosphorylation were first determined. In agreement with Huijbers et al. it was found that the IgG4 fraction reduced agrin-dependent MuSK phosphorylation but not the IgG1-3 fractions (Huijbers et al. 2013). MuSK phosphorylation was restored in myotubes incubated with IgG4 subclass fractions with NSC-87877 suggesting successful modulation of the agrin/LRP4/MuSK/DOK7 signalling downstream of MuSK/LRP4 disruption.

The modulatory effects of SHP2 inhibition and MuSK-Abs on AChR clustering were next evaluated. In agreement with Zhao et al. SHP2 inhibition stimulated the formation of AChR clusters without the need for agrin (Zhao et al. 2007). For these experiments MuSK-MG sera rather than purified IgG were used as non-IgG fractions of SNMG sera (some later found to contain MuSK-Abs) have previously been shown to have functional effects on nicotinic AChR responses (Barrett-Jolley et al. 1994) MuSK-MG sera inhibited AChR cluster formation and dispersed agrin dependent and independent AChR clusters in a concentration dependent manner. SHP2 inhibition attenuated these effects, and following dispersal by MuSK-MG sera, increased the re-formation of AChR clusters. Importantly the increase in quantity of AChR clusters did not appear to be due to the formation of new micro-clusters, as size of AChR clusters was also increased with SHP2 inhibition. However, a possible limitation of these findings was that the automated image analysis of AChR clusters excluded regions of interest smaller than 3 μ m. Calibration of the image analysis was checked with

each experiment and although regions of this size were commonly due to artefact, micro-clusters may have been inadvertently excluded. Reassuringly the analysis was sufficiently sensitive to detect that the increase in AChR cluster size was not as consistent at higher dilutions of MuSK-MG sera in DOK7 overexpressing myotubes. These myotubes have high levels of basal MuSK phosphorylation and spontaneously form AChR clusters (Beeson et al. 2006). One possible explanation for this finding is that SHP2 inhibition produces a saturating effect on MuSK phosphorylation that leads to downregulation of MuSK receptors and/or kinase activity, as has been shown for other receptor tyrosine kinase receptors (Lemmon & Schlessinger 2010).

Interestingly, inhibition of MuSK phosphorylation was not seen with the total IgG fraction from MuSK-MG plasmas. An important factor in determining the reason for this was not assessed; that is whether MuSK-IgG1-3 antibodies, which are divalent, are able to induce MuSK phosphorylation in an agrin independent manner. With equal amounts of IgG1-3 and IgG4 according to protein concentration, a reduction in MuSK phosphorylation would be expected, due to the higher MuSK-Ab concentration of the latter.

Shigemoto et al. have previously shown that MuSK-Abs from mice immunised with the MuSK ectodomain induce agrin independent MuSK phosphorylation but reduce AChR clustering in myotubes, and such models result in the production of monospecific divalent antibodies akin to human IgG1-3 Abs (Shigemoto et al. 2006; A. M. Gomez et al. 2010). IgG1-3 fractions from MuSK-MG plasmas also disperse agrin dependent and independent AChR clusters *in vitro*. Collectively, these data suggest that simply phosphorylating MuSK may not be sufficient to induce AChR clustering, and in some cases could be detrimental. It may be that there are MuSK phosphorylation sites that negatively regulate its function, indeed it is recognised that serine phosphorylation and tyrosine phosphorylation have opposing effects in the regulation of glycogen synthase kinase-3 (GSK-3), of which the β -isoform is involved in the capture of microtubular plus-ends and AChR clustering (Basu et al. 2014). MuSK-IgG1-3 Abs could phosphorylate but prevent release of MuSK kinase auto-

inhibition, resulting in incomplete phosphorylation of the tyrosine residues necessary for full MuSK activation. The latter could also affect phosphorylated Tyr 553, the binding site for the PTB (phosphotyrosine binding) domain of DOK7. Interestingly Ammar et al. have reported a M835V missense mutation in the MuSK kinase domain in a patient with congenital myasthenia gravis. This mutation resulted in a high level of basal MuSK phosphorylation, and when expressed in HEK-293 cells impaired co-immunoprecipitation of DOK7 with MuSK (Ammar et al. 2013). Therefore it would also be important to assess the effects of IgG1-3 fractions from MuSK-MG patients on agrin dependent DOK7 phosphorylation, MuSK phosphorylation in DOK7 overexpressing myotubes, and co-immunoprecipitation of MuSK and DOK7.

The experiments to assess the effects of SHP2 inhibition on AChR clustering were performed with at least 13 MuSK-MG sera for each paradigm tested. Where possible samples were selected such that a broad range of MGFA and MuSK-Ab concentrations were included. However, due to the limited volumes available substitution of some sera was required and each paradigm was only tested once. The use of an automated image analysis macro for the quantification of AChR clustering was time efficient as compared to manual counting, and allowed for blinding throughout the experiment. However, as mentioned earlier, during calibration, setting of thresholds for size and background fluorescence were necessary to reduce the detection of artefact. This varied between experiments and prevented the accurate assessment of regions of interest less than 3µm in size.

These experiments provide support the hypothesis that the consequences of MuSK/LRP4 disruption by MuSK antibodies can be ameliorated by modulation of the agrin/LRP4/MuSK/DOK7 pathway, specifically MuSK phosphorylation.

Chapter 6: Discussion

6.1 Introduction

The diagnosis of myasthenia gravis is predominantly clinical, but serological testing for autoantibodies can be useful. The clinical profile of patients detected by newer assays such as CBAs is unclear and negative sera may contain antibodies to novel antigenic targets.

An international cohort of 250 MG patients was systematically tested by RIAs and/or CBAs for AChR, MuSK, and LRP4-Abs. The MuSK CBA required optimisation to detect IgG-Abs specifically, and the potential relevance of these Abs were confirmed in AChR clustering assays. Antibodies were subsequently detected in 17% (39/231) sera negative by RIAs. Notably clustered-AChR and triple-SNMG patients had a poor prognosis. Five triple-SNMG sera bound to human skeletal muscle cell lines, but no novel antigenic targets were identified through immunoprecipitation and mass spectrometry. MuSK-IgG CBA positive patients had a milder clinical course and similar prognosis to MuSK-MG patients (identified by RIAs). The predominance of MuSK-IgG4 Abs was confirmed in MuSK-MG patients although MuSK-IgG 1-3 Abs were also frequently detected. To specifically target the MuSK-Ab mediated inhibition of the agrin/LRP4/MuSK/DOK7 pathway and MuSK phosphorylation, a SHP2 tyrosine phosphatase inhibitor was tested. A decrease in agrin-dependent MuSK phosphorylation was confirmed C2C12 myotubes with purified IgG4 fractions from MuSK-MG plasmas, and this was restored with SHP2 inhibition. The inhibition of agrin-dependent AChR cluster formation was confirmed with MuSK-MG samples and this effect was also attenuated by SHP2 inhibition. Furthermore

SHP2 inhibition blocked MuSK-Ab dispersal of agrin-dependent and independent AChR clustering, and increased re-formation following dispersal. The latter results suggest that targeted modulation of the agrin/LRP4/MuSK/DOK7 pathway may provide a novel treatment approach for MuSK-MG and perhaps other MG subtypes also.

Initially with the MuSK CBA, non-specific IgM bound to MuSK was detected in 11% and 19% of healthy and disease control samples respectively. Using an unlabelled secondary antibody specific for the Fc(γ) chain of IgG, and applying a tertiary fluorescent antibody for detection, MuSK-Ab detection was increased in MuSK-MG sera control sera, whilst avoiding IgM detection. The improved sensitivity may have in part been due to an amplification effect of the tertiary antibody layer. In addition, although IgM was not disease specific it was present in 11% (8/71) of MuSK-MG sera. The IgM bound to MuSK in MG sera was specific for MuSK by colocalisation and preadsorption experiments. IgM is normally in the form of pentamers with up to ten binding sites. One possible explanation for IgM detection in these assays could be the non-antigen specific binding of IgM pentamers to certain types of protein expressed at high levels on cells. Since similar problems have been found with myelin oligodendroglial (MOG) antibodies, it may be due to the extracellular immunoglobulin-like domains and/or glycosylation state of these particular proteins, and suggests caution should be used regarding the use of anti-IgG(H+L) for detection of IgG binding to similar antigens. Interestingly non-IgG fractions purified from SNMG sera, (some later found to contain MuSK-Abs) inhibited nicotinic AChR responses in TE671 cells (Barrett-Jolley et al. 1994). However, these effects may have been due to non-MuSK-IgM components in the IgG flow through fraction. Furthermore they may not have been MG-specific as similar findings have also been noted with agglutinating proteins such as concanavalin A and wheat germ agglutinin (Li et al. 1996). Importantly, MuSK-IgG but not MuSK-IgM containing sera inhibited agrin-dependent AChR clustering in myotubes. Although this does not exclude the possibility that MuSK-IgM may act on MuSK function *in vivo*, the lack of disease specificity argues against it. Unfortunately limited volumes of sera

precluded the assessment of IgG subclass. It would have been ideal to determine if MuSK-IgG positive sera consist mainly of IgG4 Abs that can inhibit co-immunoprecipitation of MuSK and LRP4, as has previously been shown with purified IgG4 fractions from MuSK-MG sera (Koneczny et al. 2013; Huijbers et al. 2013).

Overall, clustered AChR-Abs were detected in 10% (23/231), MuSK-IgG Abs in 7% (15/212), and LRP4-Abs in 1% (3/229) of MG patients negative by RIAs. Ocular MG accounted for 47% (7/15) of patients with clustered AChR-Abs. As expected there were high response rates to acetylcholinesterase inhibitors (83%) and immunotherapies (93%). In spite of this, only 18% (3/17) of patients attained remission. This contrasts with the high remission rates (50%) that we previously reported in patients mainly under the age of 18yrs (Rodriguez Cruz et al. 2015). The median age of patients with clustered AChR-Abs here was 42 (6-78), and suggests the prognosis in older patients may be worse. Triple-SNMG patients also had similarly low rates of remission (18%). Low rates of remission in South African triple-SNMG patients have previously been reported (Huda et al. 2016), and although some of those patients were included here, the results suggest that the absence of a detectable antibody maybe a more widely applicable poor prognostic factor.

As noted above, despite the use of RIAs and CBAs, the majority of patients did not have detectable AChR-Abs, MuSK-Abs, or LRP4-Abs. This contrasts with other studies, where for instance clustered AChR-Abs were found in 66% of patients negative by AChR- and MuSK-RIAs (Leite et al. 2009). One possible explanation for the low detection rates found here, was that triple-SNMG patients had an alternative diagnosis to MG, but this was unlikely, as the clinical features were similar to antibody-positive MG and beneficial responses were seen with cholinesterase inhibitors and/or immunotherapies. The majority of archived sera available were from patients who had received prior treatment with immunotherapies, and this may account for some of the differences. The detection of AChR-Abs is known to be higher in generalised MG (~80%) as compared to ocular MG (~50%; Lindstrom et al. 1976; Vincent 2014). Of the triple-SNMG patients described here,

20% (19/94) had ocular MG, whereas all the patients reported by Leite et al. had generalised MG (Leite et al. 2008). Despite these possible explanations, the low detection rate suggests there is scope for improvement in existing antibody detection methodologies. The first three B-propeller domains of LRP4 are involved in the interaction with the first Ig-like domain of MuSK, an interaction that can occur independently of agrin. In addition LRP4 self associates (Zhang et al. 2011), therefore, co-transfection of MuSK and LRP4 in a CBA may enhance the detection of antibodies to both these receptors. In addition the chaperone protein Mescd2 (mesoderm development candidate 2), which promotes the glycosylation and cell surface expression of LRP4, could also be incorporated (Hoshi et al. 2013). These strategies may help in the detection of antibodies against known antigenic targets.

It was possible that triple-SNMG sera contained antibodies to as yet, unidentified antigenic targets. Interestingly 19% (3/16) of triple-SNMG sera inhibited agrin-dependent AChR clustering in myotubes. This may suggest that these sera contain undetectable MuSK-, LRP4-, or agrin-Abs that exert a functional effect on the agrin/LRP4/MuSK/DOK7 pathway. However, despite these sera being negative by AChR-RIA and clustered AChR CBAs, experiments to assess surface AChR levels were not performed; therefore AChR internalisation could not be excluded. Similar experiments could be performed in further triple-SNMG sera, which may define a population in which novel antigenic targets are more likely to be found.

Here, a modified cell-based assay using TE671 and CN21 cells was established to enable screening for novel antigenic targets. Although this technique allowed for rapid screening of a large number of sera, fluorescent activated cell sorting may have been a more sensitive method, and has previously been described (Blaes et al. 2000). As expected AChR-Ab control sera bound more strongly to CN21 cells which express the adult AChR isoform and have higher levels of surface AChRs (Beeson et al. 1996; Beeson et al. 1996). However, despite several optimisation strategies a low proportion of positive control AChR-Ab (3/10) and MuSK-MG control sera (3/4) sera bound to TE671 and/or CN21 cells. In hindsight,

one strategy that was not assessed was the transfection of TE671 and CN21 cells with rapsyn, which may have clustered surface neuromuscular junction proteins in addition to AChRs, and improved detection rates. Three triple-SNMG sera did bind to TE671 and CN21 cells but these experiments were performed prior to MuSK CBA optimisation, using an anti-human IgG (heavy and light chains) secondary antibody. Therefore, IgM or an alternative immunoglobulin isotypes may have been responsible for the observed positivity.

The immunoprecipitation and mass spectrometry methodology allowed for the confident detection of MuSK from TE671 cells using a MuSK-MG control serum. Disappointingly, though perhaps not unsurprisingly no plausible MG candidate antigens were identified from the immunoprecipitates of the three triple-SNMG sera. Multiple proteins of the survival motor neuron complex were identified from the serum of one patient with co-existent polymyositis, and has been previously reported (Satoh et al. 2011). Though this result was of interest it did suggest that the immunoprecipitate could be further enriched for cell surface proteins. Introducing a surface biotinylation step prior to serum incubation, and performing a two-stage immune-capture, firstly with streptavidin and then with protein G could achieve this.

For the successful immunoprecipitation of MuSK at least 3x T175 flasks of TE671 cells were required, and was based on a detection threshold of 40ng of MuSK as calculated previously (Littleton et al. 2009). For these experiments, 6x T175 flasks of TE671 or CN21 cells were used, and suggests that ultimately it may be worthwhile exploring alternative sources such as the immortalised human skeletal muscle cell line- HMCL-7304 or ideally human stem cell derived co-cultures of motor neurons and skeletal muscle (Rokach et al. 2013; Guo et al. 2011).

For functional studies involving SHP2 inhibition an Italian MuSK-MG cohort was used and accordingly, the clinical and serological characteristics of these patients was first studied. This also provided the opportunity to compare the phenotype of MuSK-MG with MuSK-IgG CBA positive patients. Similar to previous reports (Evoli et al. 2003; Guptill et

al. 2011), MuSK-MG patients were mainly young females with severe bulbar and respiratory involvement. Cholinesterase inhibitors were often ineffective or poorly tolerated (20%), but responses to immunotherapy were good (97%), and remission rates were high (38%). MuSK-IgG CBA positive patients had a less severe disease course than MuSK-MG sera patients, and interestingly 75% (6/8) of patients improved with cholinesterase inhibitors. One group has reported that MuSK-Abs disrupt the interaction between MuSK and Collagen-Q, and suggested that this may account for the ineffectiveness and/or intolerance of cholinesterase inhibitors in MuSK-MG (Kawakami et al. 2011). Co-immunoprecipitation experiments with MuSK and collagen-Q using MuSK-MG or MuSK-IgG CBA positive sera would be of interest, and may provide an explanation for the differences in treatment response between these subgroups.

MuSK-IgG4 was confirmed as the predominant IgG subclass in MuSK-MG and as has previously been noted, correlated with disease severity (Niks et al. 2008). A higher proportion of patients with low MuSK-IgG4 or MuSK-Ab/nM titres attained remission, though sampling of most patients had occurred after immunotherapy and was a confounding factor. MuSK-IgG 1-3 Abs were also frequently detected, and in a proportion of sera (29%) at higher titres than MuSK-IgG4-Abs. Interestingly MuSK-IgG2 end-point titres correlated with disease severity. However, previous passive transfer of MuSK-MG IgG1-3 fractions did not cause a myasthenic phenotype in mice. IgG4 fractions contain approximately 100 fold higher concentration of MuSK-Abs/mg as compared to IgG1-3 fractions (Koneczny et al. 2013). As the amounts of IgG4 and IgG1-3 fractions used in these experiments were based on the total protein rather than MuSK-Ab concentration, the lack of effect of IgG1-3 fractions may have been due to an insufficient concentration of MuSK-Abs. Indeed MuSK-Abs were only detectable in animals passively transferred with MuSK-IgG4 (Klooster et al. 2012). The MuSK-IgG1-3 fraction may well be pathogenic as they disperse both argin dependent and independent AChR clusters *in vitro* (Koneczny et al. 2013).

MuSK-MG is usually treated with steroids and other forms of immunosuppression such as rituximab (Evoli et al. 2003; Guptill et al. 2011; Evoli et al. 2008; Keung et al. 2013). Whilst these therapies are effective, they are associated with significant side-effects and do not target effector mechanisms of MuSK-Abs specifically. Most MuSK-Abs are of IgG4 Abs which inhibit the agrin/LRP4/MuSK/DOK7 pathway (Huijbers et al. 2013; Konecny et al. 2013). In addition Huijbers et al. demonstrated that IgG4 from two MuSK-MG patients inhibited MuSK phosphorylation. To specifically modulate this key-signalling event, inhibition of SHP2, a tyrosine phosphatase that negatively regulates MuSK phosphorylation was tested. IgG4, but not IgG1-3 fractions from MuSK-MG plasmas inhibited agrin-dependent MuSK phosphorylation and this was restored with SHP2 inhibition. Interestingly, inhibition of MuSK phosphorylation was not seen with the total IgG fraction from MuSK-MG plasmas. MuSK-IgG1-3 Abs, which are divalent, may induce MuSK phosphorylation in an agrin-independent manner. However, with whole IgG, a reduction in MuSK phosphorylation would be expected, due to the higher MuSK-Ab concentration in the total IgG4 fraction. Specific experiments to assess the effects of IgG1-3 fractions from MuSK-MG patients on MuSK phosphorylation with and without agrin, and with differing amounts of IgG4 fractions would help to clarify this (Cao, Huda et al. in preparation).

SHP2 inhibition also restored AChR cluster formation, blocked dispersal and increased reformation following dispersal of AChR clusters by MuSK-Abs. These results suggest that targeted modulation of the agrin/LRP4/MuSK/DOK7 pathway may represent a novel therapeutic strategy in MuSK-MG. Orally bioavailable SHP2 inhibitors are now available (Garcia-Fortanet et al. 2016) and could be tested in previously established MuSK-MG passive and active transfer models (Viegas et al. 2012). Importantly conditional knockout of SHP2 in skeletal muscle did not compromise the formation and maintenance of the NMJ, suggesting that SHP2 inhibition, at least in skeletal muscle, could be tolerated (Dong et al. 2006). Interestingly SHP2 is also involved in several oncogenic signalling pathways (Ras/Erk, PI3K, AKT, and JAK-STAT), and pharmacological inhibition has

demonstrated efficacy in the treatment of tumour xenograft and glioma mouse models (Bunda et al. 2015). These studies may help expedite the assessment and safety of SHP2 inhibitors in humans. It would also be of interest to assess the effects of SHP2 inhibition in other forms of MG, such as AChR-MG and congenital myasthenic syndromes. However, SHP2 inhibition would not address AChR internalisation by AChR-Abs, and may not be effective in CMS syndromes associated with loss of function mutations in signalling proteins downstream of MuSK e.g. DOK7 mutations (Beeson et al. 2006). In the case of DOK7 mutations, targeting negative regulators of the agrin/LRP4/MuSK/DOK7 pathway that are further downstream may be of benefit, such as Calpain, β -Catenin, Nestin or GSK-3 β . Lastly, the functional inhibitory effects of IgG4 Abs in MuSK-MG may not be a unique. There are an expanding number of recognisable IgG4 antibody mediated conditions (Huijbers et al. 2015), and it maybe that the effector mechanisms of IgG4 Abs in some of these conditions are suitable for similar targeted therapies.

Publications Generated

1. Huda S., et al., 2017 An IgG-specific cell-based assay detects potentially pathogenic MuSK-Abs in seronegative MG. *Neurol Neuroimmunol Neuroinflamm* (Accepted)
2. Huda S., et al., 2016. Characteristics of acetylcholine-receptor-antibody-negative myasthenia gravis in a South African cohort. *Muscle and Nerve*. 54(6),pp.1023-1029
3. Rodriguez P. M., Al-Hajjar M., Huda S., et al., 2015. Clinical features and diagnostic usefulness of antibodies to clustered acetylcholine receptors in the diagnosis of seronegative myasthenia gravis. *JAMA Neurol*, 72(6), pp.642-639
4. Rodriguez P. M¹, Huda S¹, et al. 2015. Use of cell-based assays in myasthenia gravis and other antibody-mediated diseases. *Exp Neurol*, 270,pp.66-71
5. Wong S., Huda S., et al., 2014. Ocular myasthenia gravis: controversies and updates. *Curr Neurol Neurosci Rep*. 14(1).pp421

Appendix

Patient	Country	Sex	Age	AChEI	NSC/EMG	MGFA Onset	MGFA nadir	MGFA f/u	Worst symptom	Treatment	cAChR CBA score	Thymectomy	Immuno response	PIS
1. BID33	Ger	F	42	Y	Y	2a	2a	2a	B	AChEI,P	1	-	Y	MM
2. CIB01	Ger	F	49	-	-	2a	3b	-	B	AChEI, P,A	2	-	Y	MM
3. DID 10	Tur	F	78	Y	N	2b	3b	1	B R	AChEI	1.5	N	-	I
4. DID12	Tur	M	65	Y	N	2a	4b	0	B R	AChEI,P	2	N	Y	PR
5. DID 32	Tur	M	58	-	-	3b	4b	0	B	AChEI,P, A	1	N	Y	MM
6. FIG11	SK	F	6	N	-	1	1	1	L	AChEI	1	-	-	U
7. FIG18	SK	F	28	Y	-	1	3a	2a	L	AChEI	1	-	-	I
8. FIG30	SK	M	40	-	-	1	1	1	-	AChEI, P,A	1	-	N	U
9. RR74	Phi	-	-	-	-	-	-	-	-	-	2	-	-	-
10. JB(a)	UK	F	33	-	-	-	-	-	-	-	1.5	-	-	-
11. DB	UK	F	32	Y	N	1	1	0	O	prev ChI, P	2.5	Y -/-	Y	PR
12. SF	UK	F	42	N	N	1	1	1	O	P	1	N	Y	MM
13. EG(a)	UK	F	37	-	Y	1	1	1	O	AChEI	1	N	-	-
14. JG	UK	F	48	Y	Y	3a	4a	0	O B	A,P, AChEI	1.5	Y ^{a/y}	Y	MM
15. MH	UK	M	19	Y	Y	1	1	1	O	-	1.5	N	-	-
16. LJ(b)	UK	F	35	-	Y	1	1	1	O	AChEI, P,MMF	1.5	Y	Y	I
17. GO	UK	F	33	Y	N	3a	2a	2a	O	AChEI, P	1.5	N	Y	MM
18. 326	Ita	F	18	Y	-	-	-	2a	-	-	1.5	Y ^{h/-}	-	R
19. 598	Ita	F	54	Y	-	-	-	3a	-	-	1	N	Y	MM
20. MG1059	Nor	M	48	-	Y	-	-	3a	-	A	1.5	N	Y	I
21.MG1130*	Nor	M	44	-	Y	-	-	1	-	AChEI	1	N	-	MM
22. MG1329	Nor	M	55	-	Y	-	-	1	-	AChEI, P	1	N	Y	-
23. MG1342	Nor	-	-	-	Y	-	-	-	-	AChEI, prev P	1	-	Y	-

Table A.1. Clinical data of 23 clustered-AChR positive patients. * = LRP4-positive; NCS/EMG = nerve conduction studies/electromyography; MGFA = Myasthenia Gravis Foundation America; PIS = post intervention status; A = azathioprine; P = prednisone; AChEI; acetylcholinesterase inhibitor; CSR = complete stable remission; PR = pharmacological remission; MM = minimal manifestations; I = improved; U = unresponsive; Thymectomy (Y/N = yes/no; ^h = hyperplasia; ^a = atrophy ⁿ = normal; ^{Y/N} = improvement/no improvement following thymectomy); Ger = Germany; Tur = Turkey; SK = South Korea; Phi = Philippines; UK = United Kingdom; Ita = Italy, Nor = Norway

Patient	Gender	Onset/ yrs	MGFA at onset	MGFA at sample	PIS	Treatment	MusK RIA Titre/nM	IgG Titre 1/x	IgG1-3 Titre 1/x	IgG4 Titre 1/x
1. M080	F	53	-	0	I	A 100mg P 12.5mg	4.87	1280	50	150
2. M095	M	40	2b	0	RT	No tx	1.15	80	25	50
3. M103	M	42	3b	0	PR	P 25mg	3.42	320	25	25
4. M105	F	67	-	0	MM	P 20mg	0.73	320	75	25
5. M108	F	43	2b	0	PR	P 25mg, IVIG	7.76	1280	50	50
6. M111	F	44	4b	0	I	P 12.5mg	1.07	80	0	50
7. M112	F	16	3b	0	PR	P 17.5mg	4.62	1280	75	25
8. M116	F	32	3b	0	PR	P 17.5mg	9.65	1280	100	50
9. M123	M	44	3b	0	I	P 25mg IVIG	7.97	1280	50	25
10. M128	F	50	4b	0	PR	P 12.5mg	17.47	5120	150	200
11. M133	F	23	3b	0	I	P 12.5	6.54	1280	50	100
12. M138	F	20	2b	0	PR	P 10mg	2.13	320	25	25
13. M150	F	19	3b	0	PR	P 12.5mg	3.62	320	25	25
14. M166	F	29	2b	0	PR	P 12.5mg	2.75	320	50	25
15. M168	F	49	2b	0	PR	P 10mg	3.66	320	25	25
16. M176	F	18	3b	0	PR	P 37.5mg PLEX	4.69	1280	50	100
17. M178	F	70	5	0	MM	P 5mg	3.41	320	50	50
18. M182	F	49	2a	0	CSR	No tx	0.06	20	0	25
19. M081	F	67	3b	1	MM	P 7.5/5mg	10.11	1280	50	25
20. M087	M	61	4b	1	I	P 50mg	0.63	320	100	100
21. M092	F	28	5	1	MM	P 30mg	0.41	80	0	25
22. M097	F	24	2b	1	MM	P 40mg	12.73	20- 480	75	150
23. M129	M	37	2b	1	I	P 15mg	4.15	320	25	100
24. M149	F	36	-	1	MM	A, P, Mestinon	6.88	1280	50	75
25. M154	F	50	3b	1	MM	P 12.5mg IVIG	6.88	320	25	50
26. M083	F	19	2b	2b	PR	P 25/0mg	6.88	1280	50	200
27. M091	F	58	2b	2b	MM	MP 8mg, Mest 300mg	8.80	5120	75	200
28. M094	F	22	4b	2b	I	A 100mg, P 30mg, Mest 360mg	4.21	1280	50	100
29. M096	F	54	3b	2b	I	P 25mg, Mest 180mg	5.62	1280	25	50
30. M098	F	70	4b	2b	PR	P 12.5mg	0.25	20	0	0
31. M099	F	12	4b	2b	MM	P 5mg	13.00	5120	75	100
32. M102	F	45	3b	2b	PR	P 52.5mg, IVIG	18.00	1280	50	100
33. M107	F	40	-	2b	PR	P 25mg IVIG	0.03	80	25	0
34. M109	F	14	4b	2b	I	P 30/25mg IVIG	4.05	320	50	25
35. M110	F	19	3b	2b	PR	A 100mg, P 12.5mg	9.25	1280	50	50
36. M113	F	43	2b	2b	PR	P 10mg	2.03	80	50	25
37. M119	F	35	3b	2b	I	P 17.5mg	1.64	80	50	25
38. M122	F	40	3b	2b	-	P 25mg	9.25	1280	50	100
39. M125	F	36	5	2b	I	P 35mg	3.84	1280	50	25
40. M132	F	18	3b	2b	I	P 25/20mg IVIG	13.58	1280	100	200

41. M136	F	52	3b	2b	PR	P 7.5mg Mest 60mg	0.01	20	50	0
42. M139	M	19	2b	2b	PR	P 25mg	2.33	320	50	50
43. M147	F	33	3b	2b	PR	P 25mg	1.24	80	25	0
44. M152	F	40	4b	2b	PR	P 25mg, Mest 150mg	1.50	320	50	25
45. M153	F	26	2b	2b	PR	P 17.5mg	2.92	320	25	25
46. M155	F	46	3b	2b	I	P 25mg	9.32	1280	50	50
47. M157	F	39	3b	2b	I	P 50mg, IVIG, Mest 200mg	5.28	1280	50	50
48. M160	F	30	4b	2b	PR	A 100mg, P 12.5mg, IVIG	3.84	80	50	25
49. M161	F	26	4b	2b	I	P 62.5mg PLEX	9.32	5120	50	400
50. M162	F	82	3b	2b	I	P 50mg	0.26	20	0	0
51. M163	F	44	3b	2b	I	P 47.5mg	2.00	320	25	25
52. M165	F	49	5	2b	I	A 100mg, P 62.5mg	5.27	320	50	25
53. M169	F	40	3b	2b	I	P 12.5mg	5.27	1280	50	100
54. M170	F	38	4b	2b	MM	P 25mg	3.92	320	50	25
55. M172	F	70	3b	2b	I	A 100mg, P 20mg	7.78	1280	50	100
56. M175	F	44	3b	2b	PR	P 17.5mg	4.86	320	25	50
57. M179	F	18	2b	2b	PR	P 12.5mg Mest 180mg	12.72	1280	150	300
58. M180	F	47	3b	2b	MM	A 100mg, P 5mg	12.72	5120	125	100
59. M183	F	56	2b	2b	PR	Dex 8mg	11.72	5120	75	200
60. M186	F	23	2b	2b	MM	No tx	14.13	1280	75	150
61. M085	F	30	3b	3b	U	No tx	12.72	1280	100	200
62. M093	F	63	4b	3b	I	P 12.5mg	12.72	5120	75	150
63. M089	F	59	3b	3b	MM	Cycl 100mg, P 15mg, IVIG	10.08	5120	125	100
64. M126	F	57	3b	3b	-	A 100mg, P 12.5mg, Mest 240mg	7.97	1280	50	50
65. M145	F	37	3b	3b	U	A, P 35mg	0.91	n/s	n/s	n/s
66. M164	F	16	2b	3b	MM	Mest 150mg	17.06	5120	50	300
67. M167	F	46	2b	3b	I	No tx	9.45	1280	75	100
68. M173	F	42	3b	3b	I	P 50mg	8.57	1280	50	100
69. M174	M	56	4b	3b	MM	P 57.5mg	1.70	80	25	25
70. M159	F	28	4b	4b	I	P 50mg	24.96	5120	175	300
71. M181	F	56	3b	4b	PR	Mest 120mg	4.37	1280	50	150

Table A.2: Clinical and serological data of 71 MuSK-MG patients- MGFA = Myasthenia Gravis Foundation America; yrs = years; n/s = non-specific; PIS = post intervention status; A = azathioprine; P = prednisone; IVIG = intravenous immunoglobulin; tx = treatment; Mest = mestinon; PLEX = plasma exchange; Dex = dexamethasone; Cycl = cyclosporine; CSR = complete stable remission; PR = pharmacological remission; MM = minimal manifestations; I = improved; U = unresponsive

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