Myasthenia gravis; cytotoxic potential of human sera in vitro and novel non-antibody, disease-modifying factors

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LIST OF PAPERS

i. Luckman SP, Skeie GO, Helgeland G, Gilhus NE. Morphological effects of myasthenia gravis patient sera on human muscle cells. Muscle Nerve. 2006 Jan;33(1):93-103.

ii. Helgeland G, Luckman SP, Romi FR, Jonassen AK, Gilhus NE. Myasthenia gravis sera have no effect on cardiomyocytes in vitro. J Neuroimmunol. 2008 Sep 15;201-202:74-9.

iii. Helgeland G, Petzold A, Hoff JM, Gilhus NE, Plant GT, Romi FR. Anti-Heat Shock Protein 70 antibody levels are increased in myasthenia gravis and Guillain-Barré syndrome *(in press)*

iv. Helgeland G, Petzold A, Luckman SP, Gilhus NE, Plant GT, Romi FR. Matrix metalloproteinases in Myasthenia Gravis *(submitted)*

ABBREVIATIONS

AChE-I	Acetylcholine esterase inhibitor
AChR	Acetylcholine receptor
AMC	Arthrogryposis multiplex congenital
AZT	Azathioprine
CD	Cluster of differentiation
CMAP	Compound muscle action potential
CsA	Ciclosporin A
CSF	Cerebro spinal fluid
CT	Computer tomography
Cy	Cyclophosphamide
DC	Dendritic cells
EAE	Experimental autoimmune encephalitis
EAMG	Experimental autoimmune MG
ECC	Excitation-contraction coupling
ECG	Electrocardiogram
ECM	Extracellular matrix
EDC	Extensor digitorum communis muscle
EF	Ejection fraction
EOMG	Early onset myasthenia gravis
FDG-PET	Fluordeoxyglucose-positron emission tomography
GBS	Guillain-Barré syndrome

GMG	Generalized MG
HLA	Human leukocyte antigen
HSP	Heat shock proteins
IFN	Interferon
IgG	Immunoglobulin G
IVIG	Intravenous IgG
LOMG	Late onset myasthenia gravis
LPS	Lipopolysaccharide
MC	Myasthenic crisis
MG	Myasthenia gravis
MGFA	Myasthenia gravis foundation of America
MHC	Major histocompatibility complex
MIR	Main immunogenic region
MMF	Mycophenolate mofetil
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MuSK	Muscle specific kinase
MXT	Methotrexate
nAChR	Nicotinic Acetylcholine receptor
NMJ	Neuromuscular junction
OMG	Ocular MG

PE	Plasma exchange/plasmapheresis
RA	Rheumatoid arthritis
RIA	Radioimmune assay
RNS	Repetitive nerve stimulation
ROI	Regions of Interest
RyR	Ryanodine receptor
SFEMG	Single fiber electromyography
SLE	Systemic lupus erythematosus
SNMG	Seronegative MG
SR	Sarcoplasmic reticulum
SS	Sjögren's Syndrome
TIMP	Tissue inhibitors of metalloproteinases
WHO	World Health Organization

INTRODUCTION

Myasthenia gravis

Epidemiology

Myasthenia Gravis (MG) is a rare autoimmune disease. Reports from the past decades report an annual incidence between 4 and 11 per million [1-3], and prevalence ranging from 70 to 150 per million [1-4]. Recent data from our department confirms these numbers, estimating the annual incidence to 7.2 per million, and prevalence 145 per million [5]. Both the prevalence and incidence rate have increased during the last 40 years [6], probably due to better diagnostics technology, improved medical treatment and epidemiological case finding, and a population consisting of more elderly individuals and less children.

From generally being considered a disease affecting mainly young adults, it is now increasingly clear that the disease has a higher annual incidence and prevalence in the elderly, and is likely underdiagnosed in the very old [5, 7]. Difficulties in diagnosing this population may be due to age-related alteration of facial features, and because symptoms are believed to be caused by other neurological diseases, such as cerebrovascular disease or motor neuron disease [7-8]. MG onset also shows some sex-related characteristics. Among people below the age of 50, women are more likely to develop MG, whereas the sex ratio is near unity in the older population [5].

Clinical features

The classical MG symptoms are weakness in striated muscle, muscular fatigue and reversible exercise-induced loss of muscle strength. Any groups of muscles can be affected, and the severity ranges from confinement to only one muscle group, e.g. ocular or periocular muscles, to generalized disease affecting proximal limb muscles and head and neck musculature. Approximately 2/3 of patients have ocular or extra-ocular muscle weakness as initial symptoms. While most patients progress to a more generalized disease, some 10% of patients retain this phenotype and are categorized as having ocular MG (OMG) [9]. Dysarthria, difficulties in swallowing and alteration of facial expression can be the dominating feature in some patients with affection of bulbar muscles. Reduced ventilatory capacity has been reported in MG patients with or without dyspnoea. Weakness in

respiratory muscles predispose to serious, and sometimes fatal, respiratory failure and respiratory infections. In a small group of patients, respiratory muscles can be affected to such a degree that mechanical ventilation is needed (myasthenic crisis).

Subgroups

Patients with MG can be divided into several subgroups, according to disease manifestations, age of onset and antibody profile. The following subgroups are commonly accepted:

Ocular MG

Patients are defined as having ocular MG (OMG) if their symptoms remain purely ocular, i.e. only affecting ocular and periocular muscles, for more than 2 years. Approximately 50% of patients which start off with ocular symptoms, develop generalized disease during this period. Some have suggested that in MG patients displaying only ocular symptoms at disease onset, having undetectable levels of acetylcholine receptor (AChR) antibodies (by conventional radioimmune assay (RIA)), and also steroid-treatment reduce the risk of developing generalized MG (GMG) [10-12], but these are retrospective studies without controls. Only half of OMG patients have detectable levels of anti-AChR antibodies [13], and anti-MuSK and anti-titin antibodies are not found. Thymus pathology is rarely seen.

Early Onset MG (EOMG)

Patients developing generalized MG without a thymoma before the age of 50 are said to have early onset MG. AChR-antibodies are present in about 80% [14], and thymus hyperplasia is often found. Antibodies towards titin are found in 10%, and antibodies giving a cross-striational binding pattern on tissue sections from skeletal or heart muscle are found in 25%. Antibodies towards ryanodine receptor (RyR) are very rarely detectable. Females are over represented in this group by a factor of 2-4. Thymectomy is believed to be effective [15-16].

EOMG is strongly associated with HLA-B8*DR3 or ancestral haplotype 8.1 [17].

Late Onset MG (LOMG)

This group includes patients with onset of symptoms after the age of 50 years without a thymoma and not only ocular symptoms. Over 90% are anti-AChR positive, and antibodies

targeting other proteins (titin, RyR, IFN-α2 and IFN-ω) are commonly present. Male:female ratio is close to 1. Thymic pathology is seldom seen; hence thymectomy plays no major part in treatment [15, 18]. LOMG is associated with HLA-A3*B7*DR2 [19] and HLA-DR4 [20].

Some patients with disease onset after 50 years show features similar to EOMG, and are denoted delayed early onset. They have an increased frequency of the extended HLA-A1-B8-DR3 haplotype [21], thymic hyperplasia, and their treatment response is similar to the EOMG group [16].

Thymoma MG

10-15% of MG patients have a thymoma, making their type of MG a true paraneoplastic disease. 30-40% of all thymoma patients develop MG. All thymoma MG patients have detectable levels of AChR antibodies and a high percentage have antibodies towards titin (90%) and RyR (75%) [15]. Onset of the disease peaks around the age of 50, but can occur at any point throughout life. There is no sex difference within the group. The patients differ clinically from those in the other MG groups in that they at time of onset more often have neck weakness and non-limb involvement [22]. No clear HLA-association has been found among thymoma MG patients.

As with most other tumors, thymomas can be both benign and malign, and may show varying degrees of infiltration and invasion of surrounding tissues and structures. Surgery is the cornerstone in the treatment. Chemotherapy or radiation can be added for advanced stages of the disease, and gives a better prognosis for these patients [23-25].

Anti-MuSK MG

Antibodies towards the protein Muscle specific kinase can be detected in 5-40% of patients negative for anti-AChR antibodies. These patients almost never have antibodies towards AChR. The prevalence of anti-MuSK MG seems to be associated with geographical latitude, as only three patients are known in Norway (two ethnic Norwegians and one Pakistani woman), whereas reports from UK, Denmark, Poland and the Netherlands indicate a 15-20% prevalence, and in the Mediterranean region up to 40% [26]. MuSK-MG patients often have a more severe MG with prominent bulbar and upper body muscle involvement. There is a strong female preponderance, and muscle atrophy can be seen [27-28]. Although the anti-MuSK subgroup show characteristic features, clinically it is impossible to distinguish this

subgroup from the other groups for the individual patient [16]. The response to acetylcholineesterase inhibitors (AChE-I) is reduced compared to the other MG groups [27].

MuSK antibody positive MG is strongly associated with HLA-DR14-DQ5 [29].

MG with low affinity antibodies to AChR

In patients with generalized MG over 80% are positive for anti-AChR antibodies when using conventional radioimmune assays (RIA). Of the remaining 20%, 5-40% are positive for anti-MuSK, whereas the rest were listed as seronegative. A report from Leite et al. showed the presence of low-affinity antibodies towards AChR when the protein is over-expressed and clustered in stable, non-muscle cell lines [30]. Over half of the patients previously listed as seronegative had these antibodies, which were capable of activating complement. These findings strongly indicate that a proportion of the patients previously categorized as seronegative, in fact have pathological antibodies. This is further supported by the observation that these patients are more similar to the anti-AChR patients than to the anti-MuSK group, both regarding clinical features and response to treatment [30].

Seronegative MG (SNMG)

The method utilized in [30] is quite labor intensive compared to the standard method for measuring anti-AChR antibodies. It requires co-transfection of several genes into a eukaryotic cell of human origin, and a manual evaluation of the test by means of immunofluorescence. This makes it difficult to standardize, and the conventional anti-AChR RIA remains the method of choice. As a result the MG group denoted seronegative MG has to be viewed as a heterogeneous group consisting of both anti-AChR positive and –negative patients. This heterogeneity makes it difficult to interpret studies which use the presence of anti-AChR antibodies as a grouping variable.

This group comprises only 10-20%, of MG patients, and has decreased in size due to the discovery of low affinity anti-AChR antibodies [30]. These patients have in their sera no detectable antibodies against AChR and MuSK. Improvement with plasmapheresis, reduced number of AChRs on the muscle endplate and the observation that immunoglobulin from patients can transfer the disease to animals support SNMG as an antibody-mediated disease. Non-antibody factors may also play a role. The lack of detectable antibodies makes the diagnostic process more challenging as neither electrophysiological examinations nor

response to edrophonium have the same sensitivity and specificity as the anti-AChR assay. The patients are clinically heterogeneous. There seem to be subtle clinical differences between seropositive and seronegative MG, but these are difficult to quantify [31].

Pathophysiology

The developmental process of the neuromuscular junction (NMJ) is not known in great detail. Agrin, a proteoglycan secreted from the nerve terminals, play a crucial role in clustering of the nicotinic AChRs (nAChR). Anchoring of these clusters on the muscle membrane is reached through agrin/MuSK interaction and expression of the protein rapsyn (receptor associated protein of the synapse), but the biochemical specifics concerning this process remain unclear [32].

AChRs are part of a superfamily of pentameric neurotransmitter-gated ion channels. Each channel consists of five homologous subunits organized around a central ion channel. cDNA from 16 receptor subunits have been cloned from several species: nine α-subunits, four β-subunits and one δ-, γ- and ε-subunit [33].

There are two types of muscle nAChRs, the fetal and the adult form. The fetal type, with the composition $(\alpha 1)_2\beta\gamma\delta$, is expressed during embryogenesis and is replaced by the adult isoform after innervation. However some muscles, notably the extraocular muscles, still express fetal AChR [9]. Adult nAChR differs from fetal nAChR in that a ε-subunit replaces the γ-subunit. Although the mechanism for this γ/ε-subunit shift is not known in detail, it is clear that ACh-mediated muscle activity stimulates the shift, and that both subunits are essential for normal muscle development and innervation [32].

In most cases, MG is caused by autoantibodies binding to the nAChR on the post-synaptic membrane in the neuromuscular synapse. More than half of the autoantibodies are directed towards a conformation-dependent region called the main immunogenic region (MIR) on the α1-subunit. Binding to this region allows for the cross linking of two adjacent AChRs [33].

Anti-AChR antibodies found in MG can bind to both fetal and adult form of nAChRs. Antibodies targeting the γ-subunit will not give symptoms in adults, but can cause

congenital malformations and muscle weakness, characteristic of arthrogryposis multiplex congenita, in children of antibody-positive mothers [33].

Commonly, three antibody-mediated pathophysiological mechanisms are recognized in anti-AChR positive MG [9]:

1. Antibodies bind to the AChRs in the NMJ and activate the complement system. This leads to localized membrane destruction, ultimately causing an altered NMJ-morphology with reduced height of the post-synaptic membrane folds and a widened synaptic cleft.
2. AChRs are cross-linked and endocytosed and degraded by the muscle cell, a process known as antigenic modulation. This increased receptor turnover leads to decreased amount of receptors on the cell surface.
3. The anti-AChR antibodies block the agonist binding site, preventing ACh-mediated depolarization of the post-synaptic membrane.

Patients with anti-MuSK almost never have antibodies towards AChR in their sera, and their disease is considered to be an immunologically separate form of MG. In animal studies, anti-MuSK inhibits agrin-mediated AChR-clustering in vitro and reduces postsynaptic AChR levels in vivo [34]. The antibodies also have an inhibiting effect on regenerating end-plates [35].

Apart from anti-AChR and anti-MuSK antibodies, a variety of other antibodies targeting different muscle proteins have been detected in MG-patients [36-39]. RyR is an ion channel in the sarcoplasmic reticulum (SR) of skeletal muscle cells, and releases calcium into the cytosolic space upon sarcolemma depolarization and activation of the dihydropyrimidine receptor [40]. MG-associated anti-RyR antibodies are capable of inhibiting RyR-function in vitro, when the protein is directly exposed to antibodies [41], but their ability to block function in live cells or in vivo have not been shown. MG patients also have prolonged excitation-contraction coupling (ECC) time [42]. In vitro studies on MG muscle biopsies show non-AChR mediated changes in ECC [43]. These findings indicate that mechanisms beside AChR-defects play a role in disease pathogenesis.

Disease severity correlates with antibody levels towards titin. Titin is a huge filamentous protein, third most abundant in muscle, and functions both as a scaffold for the sarcomere

unit and as a spring to give skeletal muscle its biomechanical properties during contraction and relaxation [44].

The occurrence of anti-RyR and anti-titin antibodies is most frequent in patients with late onset or thymoma MG. Both antibodies are associated with disease severity [45], but no direct evidence has been demonstrated for their role in MG pathogenesis. Both proteins are intracellular proteins, and should therefore be “unreachable” for circulating antibodies, though antibody internalization has been described [46].

The association between MG and thymus and thymoma has been known for a century. The thymus has a crucial role in development of the immune system, as congenital absence of the thymus causes life-threatening immunodeficiency [47]. The thymus is central in T-cell development. $CD4^-CD8^-$ cells, which are believed to be primitive T-cell precursors [48-49], mature into $CD4^+CD8^+$ cells before attempting to rearrange their T-cell receptor genes. If successful, the cell proceeds to two stages of selection, where the cells ability to recognize self-MHC molecules (positive selection, which takes place in the cortex), and not recognize self-proteins (negative selection, which takes place in the medulla) is tested, before the cell is released from the thymus into the circulation [47, 50].

According to WHO’s classification, thymomas are divided into group A, group B and the mixed AB group. Type A has a medullary appearance, whereas type B exhibits features of the thymic cortex, indicating that the tumor originates from the cortical epithelium of the thymus [51]. The observation that MG is more frequently associated with B-type tumors indicates that thymic structure is important in the pathogenesis of thymoma MG [50]. Furthermore, MG is associated with a high $CD4^+CD8^+$ count [50], a cell type commonly found in type B thymomas [51]. The generation of self-reactive T-cells may thus be explained by the lack of a functional antigen-presenting medullar structure with loss of intrathymic negative selection as a consequence.

Another theory with less emphasis on thymic structure explains T-cell selection through T cell receptor (TCR) -avidity and -affinity, in which the positive and negative selection relies on both the number of TCRs binding to its antigen-MHC-II counterpart (avidity) and the strength of the binding (affinity). According to this model, no or weak TCR binding will not provide the cell with sufficient signals to survive, whereas too strong binding (above an

undefined threshold) to self-peptide-MHC-II will trigger apoptosis. The expression of HLA-DR in type B thymomas is lower than in the normal thymus, and could theoretically cause a T-cell selection-shift allowing self-reactive T-cells into the circulation [50].

B-cells don't have the same level of quality control as T-cells. B-cell activation, by most protein antigens, and subsequent antibody production, requires an additional stimulus from helper T-cells (T_H). Failure to produce this second stimulus causes the B-cell to die, a process known as clonal deletion [47]. MG patients have $CD4^+$ T_H-cells in their circulation, and patients experience improvement after treatment with anti-CD4 antibodies. The role of $CD4^+$ T_H-cells has been demonstrated in experimental systems where CD4 deficient mice failed to develop experimental autoimmune MG (EAMG). It has also been shown that $CD4^+$ T_H-cells specific for AChR-epitopes can induce anti-AChR antibody production and MG-symptoms [9].

Diagnosis

Patient history

MG patients typically present with weakness in voluntary muscles which worsen through the day and during exercise. Symptoms from ocular or extraocular muscles, such as diplopia and ptosis are common, but symptoms can arise from bulbar muscles with dysphagia, dysphonia or dysarthria, or present with respiratory difficulties. The bulbar symptoms can easily be misdiagnosed as other neurological diseases, e.g. cerebrovascular disease, especially in the elderly. Differential diagnoses include upper motor neuron disease, stroke, polymyositis, multiple sclerosis and unspecific fatigue.

Clinical examination, MGFA-score

The main objective when examining the patient suspected to have MG is to confirm the patient's sensation of fatigue and muscle weakness as objectively detectable and reproducible, representing the type of paresis found in MG patients. The examiner should perform a ptosis-test, in which the patient is to gaze upwards for more than 30 seconds while the examiner notes any signs of ptosis or gaze deviation. Symptoms from proximal limb muscles can be tested when the patient extends an arm or a leg, and tries to maintain the position of the limb for a period of time. Dysphonia and dysarthria, if present, will often

reveal itself while taking the patient's medical history. Potential respiratory involvement should be thoroughly sought for.

After having concluded upon the MG diagnosis, the patient's symptoms should be graded according to the Myasthenia Gravis Foundation of America (MGFA) classification scale [52].

Pharmacology

The pharmacological assessment of MG relies on the rapid increase in muscle strength after intravenous administration of the acetylcholine esterase inhibitor edrophonium (Tensilon). Effects of the drug occur within seconds to minutes and wear off after about an hour. The patients usually report symptom relief, but the observer should be able to objectively observe an improvement to confirm the test as positive for MG. The test is important in the assessment of MG, with sensitivity and specificity rates of 0.92 and 0.97, respectively, for OMG and 0.88 and 0.97 for GMG [53]. An alternative pharmacological approach to the diagnosis of MG is initiating per oral treatment of a patient suspected of having MG with pyridostigmine, and to assess the symptoms after a few days.

Antibody-testing

Around 85% of MG patients have antibodies towards AChR, and the MG diagnosis traditionally rests quite heavily on the results from testing for these antibodies. The antibody assay most often used is a radioimmune assay (RIA) using AChRs labeled with 125I-alpha-bungarotoxin, where immune complexes are precipitated by secondary anti-human IgGs [54].

Several studies, of varying quality, have examined the sensitivity and specificity of such tests, and have been reviewed by Benatar [53]. Generally, the sensitivity estimates in the higher quality studies are lower than in the studies of poor quality. The specificity is equally high (0.97-0.99) in all studies, irrespective of study design or whether the patients had OMG or GMG. For OMG, the pooled estimate of sensitivity ranged from 0.44 to 0.66, and in GMG from 0.90 to 0.96. This clearly shows that both a positive and a negative test is useful in assessment of potential MG, but also that a positive test is more helpful than a negative one when OMG is suspected [53]. All patients with a negative test for anti-AChR and still suspicion of MG should be tested for anti-MuSK antibodies.

Reports on low-affinity anti-AChR antibodies in MG patients previously thought to be seronegative have been published [30], but the method for this test has not yet been standardized, and is not a part of routine diagnostic work-up.

Antibodies against titin are found in 95% of thymoma MG patients, but only have a specificity of 39% for a MG patient having a thymoma. Anti-RyR antibodies are detected in 14% of MG sera, but are only found in the late onset and thymoma MG groups [15]. Together, this makes the detection of these auto-antibodies a valid supplementary tool to image diagnostics in deciding whether the patients should be thymectomized or not. The production of titin antibodies is not dependent on the pathogenic actions of anti-AChR antibodies, as they do not appear in sera from patients with long-standing, early onset MG and thymus hyperplasia [21]. RyR antibodies are in some MG patients able to inhibit calcium release from isolated terminal cisternae fractions [41], but a direct role in pathogenesis is yet to be discovered. Both titin and RyR antigens are intracellular, and no studies have shown that antibodies can bind to these proteins in vivo.

Neurophysiology

Neurophysiological tests in the assessment of MG include repetitive nerve stimulation (RNS) and single fiber electromyography (SFEMG). RNS at 2-5 Hz shows a decremental response in MG patients. This is due to depletion of the immediate ACh stores at the NMJ and a reduction of the safety factor, thereby a reduced probability of neuromuscular transmission. The test is considered positive when the fourth compound muscle action potential (CMAP) decreases more than 10%. The sensitivity and specificity of the test depends on which muscles are tested, the technique of the operator and whether the patient uses AChE-Is [55]. SFEMG is considered the most sensitive diagnostic test for detecting abnormal muscle transmission. It is time consuming and technically demanding. The method is based on simultaneous recording of muscle fiber action potentials from two adjacent muscle fibers. The difference in time between the firing of one muscle fiber action potential compared to the other is termed neuromuscular jitter. Preferably, an affected muscle should be tested. In many centers, musculus extensor digitorum communis (EDC) is initially examined, thereafter proceeding to a facial muscle if the results from EDC are normal [55]. SFEMG has a lower sensitivity and specificity in OMG than in GMG. A systematic review of electrophysiological methods in MG diagnostic work up concluded with a sensitivity of 75% to 98%, and

specificity ranging from 96% to 98% for GMG. For OMG the numbers were 62% to 97% and 73% to 96%, respectively [53].

Imaging

Patients that are diagnosed with MG or are suspected of having the disease should undergo radiological examination of the anterior chest cavity to look for abnormalities of the thymus. In MG, differentiating thymic hyperplasia and thymoma from the normal thymus is of main importance. Small anterior intramediastinal masses that do not distort the mediastinal contours will not be detectable by chest radiography [56].

Contrast enhanced CT imaging is considered equal or superior to MRI for the assessment of thymic masses, except in the cases of thymic cysts. MRI can give additional information when locally advanced disease with infiltration of surrounding organs is suspected and CT gives equivocal information [56-57]. FDG-PET can be used to distinguish between different types of thymic masses and to evaluate the presence of metastatic disease [58]. MRI with chemical shift uptake can be useful in deciding whether the patient has a hyperplastic thymus or thymoma, but its ability to discriminate pathological and healthy thymi remains unclear.[59]

Treatment of MG

Pharmacological

Drug treatment of MG should take into account the extent of symptoms the patient is experiencing as well as progression or remission of symptoms. Initial treatment for all MG subgroups is acetylcholine esterase inhibitors (AChE-I), of which pyridostigmine is the most widely used. This inhibits the breakdown of ACh in the NMJ, leading to increased levels of ACh, and increased chances of receptor binding and postsynaptic depolarization. AChE-I treatment is only symptomatic, and is most helpful when used in newly diagnosed MG and as long term treatment of mild disease [60]. Adverse effects include gut hypermobility, hyperhidrosis, excessive respiratory and gastrointestinal secretions and bradycarida, and are caused by the increment of ACh both in nicotinic and muscarinic synapses. Optimal dosage is determined by the balance between improvement in muscle strength and degree of adverse effects [60].

Corticosteroids

In many MG patients, treatment with AChE-I alone is not sufficient, and coadministration of immunosuppressants is necessary. Oral prednisolone is considered a first-line immunosuppressive treatment [60]. Recommended starting dose is 10-25mg on alternate days, and with a gradual of increase the dose to 60-80mg on alternate days. Remission usually occurs within 4-16 weeks. The prednisolone dose should subsequently be reduced slowly to the minimum effective dose given every other day. If the starting dose is too high, some patients experience a worsening of symptoms after 4-10 days, and the treatment may precipitate a MG crisis [60].

Short term methylprednisolone pulse treatment versus placebo has been reported safe and efficient [61], but the study is small and limited to patients with moderate MG. A Cochrane review of the treatment of MG with corticosteroids concluded that corticosteroids offer significant short term benefit, and are not inferior to azathioprine or intravenous IgG (IVIG) [62].

Prednisolone, and corticosteroids in general, have a variety of side effects which limit their long term use. Adverse effects include obesity, development of Cushingoid features, hypertension, reduced glucose tolerance, diabetes mellitus, increased susceptibility to infections, osteoporosis, myopathy, peptic ulcer disease, anxiety and psychoses.

Azathioprine

Azathioprine (AZT) is a pro-drug which is converted to 6-mercaptopurine. It interferes with the purine metabolism acting as a purine antagonist, and inhibits DNA and RNA synthesis. This causes a blockade of T- and B-cell proliferation. Azathioprine is recommended to be the first choice immunosuppressant drug when treating MG patients. Therapeutic response may be delayed for as long as 12 months and maximal effect may not be seen until after 24 months. Hence a combination of azathioprine and prednisolone is often initiated before a gradual tapering of prednisolone when clinical improvement is observed [60]. A small randomized controlled trial (RCT) with 34 MG patients concluded that azathioprine used together with prednisolone reduces the maintenance dose of prednisolone, gives longer remissions and fewer side effects than when using prednisolone alone [63]. Non-randomized evidence suggests that azathioprine alone has an effect in treatment of MG

[64]. Adverse effects include bone marrow deficiency and hepatotoxicity, and calls for careful monitoring of full blood cell count and liver enzymes. Less severe side effects, such as gastrointestinal disturbances and flu-like symptoms are seen in 10%, and usually occur within the first few days of treatment [65].

Methotrexate

Methotrexate (MXT) is a folate antagonist preventing synthesis of tetrahydrofolate, which is used in the conversion of dUMP to dTMP needed in DNA synthesis. Other mechanisms of action have also been suggested [66]. Consensus guidelines recommend using MTX in patients not responding to AZT. The drug is thoroughly studied in other autoimmune diseases, but evidence with sufficient quality lacks for MG [65].

Cyclophosphamide

Cyclophosphamide (Cy) has mainly a B-cell effect and suppresses antibody production. Effects on T-cells can also be achieved, but this is dependent on timing of administration and dosage [66]. It is an alkylating agent with mutagenic properties and a relatively high risk of toxicity, and may cause bone marrow depression, kidney damage, increases the risk of infections and development of neoplasms. Evidence supporting its effectiveness in MG treatment or steroid sparing is scarce [64]. Due to its adverse effects, usage is limited to patients intolerant or unresponsive to AZT, MTX, ciclosporin A or mycophenolate mofetil [64-65].

Ciclosporin A

Ciclosporin A (CsA) forms complexes with cyclophilin which through a series of steps inhibit translocation of transcription factors involved in synthesis of IL-2, IL-4 and CD40L to the nucleus. The drug also acts on the JNK- and p38 signaling pathways triggered by antigen recognition in T-cells [66]. Randomized and non-randomized evidence demonstrates its effect in MG treatment, both used alone [67] or in combination with steroids [68-70]. Like Cy, CsA has serious side effects, including systemic hypertension and nephrotixicity, and should only be considered in patients intolerant or unresponsive to AZT.

Mycophenolate mofetil

Mycophenolate mofetil (MMF) has an immunosuppressive effect through its active metabolite mycophenolate acid by inhibiting inosine 5’-monophosphate dehydrogenase

which is the rate limiting enzyme in guanine nucleotide synthesis. Non-randomized studies on the use of MMF in MG have indicated both improvement of symptoms and a steroid sparing effect. [71-73] Randomized trials, however, have not demonstrated the same effect, possible because of a slow onset of action.[64, 74] MMF has a low frequency of adverse effects compared to Cy and CsA, and those observed are usually mild, including nausea, headache and diarrhea.[73]

FK506 (Tacrolimus)

FK506 is phosphorylated in cells to act as an inhibitor of inosine monophosphate dehydrogenase. Reversal of lymphocyte proliferation suppression by guanine suggests this is the primary mechanism of action [66]. Furthermore, it acts on the calcium-calcineurin pathway to inhibit T-cell proliferation, and potentiates ECC in skeletal muscle through modulating calcium release through RyR [60, 75]. Case-reports attribute a rapid onset of effect to the RyR-modulating properties of the drug [76]. A randomized, unblinded study demonstrated that FK506 is effective, well tolerated and reduces the need for other immunotherapy [77]. FK506 should be tried in MG patients with poorly controlled disease, in particular perhaps patients positive for anti-RyR antibodies[65].

Antibodies towards leukocyte antigens

Treating autoimmune diseases with monoclonal antibodies targeting CD20 and CD4 is a relatively new treatment option. Anti-CD20 antibodies were originally designed for use in patients with lymphoma, but effects have been demonstrated in rheumatoid arthritis (RA), systemic lupus erythematosus and multiple sclerosis (MS). It causes a rapid depletion of $CD20^+$ B-lymphocytes, probably through antibody dependent cell-mediated cytotoxicity [78]. CD4 is a part of the T-cell receptor complex which recognizes peptides presented by MHC class II molecules. Presentation of an antigen to a $CD4^+$ T-cell activates both the T-cell and the antigen presenting cell. Antibodies targeting CD4 inhibits this mode of activation, resulting in decreased immune activation [79]. Case-reports from successful treatment of MG patients with monoclonal antibodies towards B-cells (anti-CD20) [80-81] and T-cells (anti-CD4) [82] have been published. Although both reporting good outcome, more thorough research is needed before any recommendations can be given [65].

Plasma exchange and intravenous immunoglobulin G

Plasma exchange (PE) and intravenous immunoglobulin G (IVIG) share indications for use: rapidly progressing disease, preparation of patients for surgery and as an adjuvant to reduce the adverse effects of oral immunosuppressants. In plasma exchange antibodies are removed from sera by membrane filtration or centrifugation. Patients experience improvement after 2-7 days, and the effect lasts for up to 3 months [65]. Short term benefit from PE has been reported in several case series, but no adequate RCTs have been performed. IVIG works by interfering with an array of different immunoregulatory components, including costimulatory molecules, adhesion molecules, cytokines, antibodies and the complement system [83]. IVIG gives a better response than placebo in severe MG exacerbation. Any benefits of IVIG compared to PE have been addressed in a Cochrane review which concludes that of two performed RCTs, one significantly demonstrated the superiority of IVIG to PE, whereas the other did not [84]. Long term therapy with PE or IVIG for stable MG is not recommended [65].

Non-pharmacological treatment options

Weight control, training and other life style modifications are considered important, but there is no solid evidence to support the recommendation [16, 65]. Some improvement in muscle strength can be achieved through physical exercise [85], and respiratory training is also beneficial [86].

Pregnancy in MG is usually uncomplicated; however MG patients have an increased risk of delivery complications and intervention during birth. No difference in severe birth defects is observed between MG and healthy women [87]. Anti-AChR antibodies transplacentally acquired by the fetus can cause transient neonatal myasthenia and require close follow up and even AChE-Is, depending on the severity of the disease [16]. It occurs in 10-15% of MG births [88]. All women with MG should give birth at units with access to ventilatory supportive treatment for the newborn. Arthrogryposis multiplex congenital (AMC) is a severe and often fatal birth defect that can be caused by anti-AChR [88]. It is characterized by hypotonia, multiple joint contractures, ptosis and respiratory distress [89], and is noted to occur in 2.2% of MG births in Norway. The risk of developing AMC increases in siblings of an affected child either with AMC or neonatal MG, and is not dependent on the mother's clinical state. [90]

Thymectomy

Despite the lack of evidence from well-controlled, randomized studies, thymectomy is well supported by long-term evidence, and is widely used as a treatment for MG. Different surgical techniques and concomitant use of immunosuppressants makes it difficult to evaluate precisely the effect of surgical intervention. According to the most recent *guidelines for treatment of neuromuscular transmission disorders* [65], MG patients undergoing thymectomy are twice as likely to achieve medication-free remission, 1.6 times as likely to become asymptomatic and 1.7 times as likely to improve. No study has reported a significant negative effect of thymectomy. The use of thymectomy in treatment of MG relates to the disease subtype:

Ocular MG

In absence of a thymoma, thymectomy is not recommended for MG patients with symptoms limited to ocular/periocular muscles [65, 91].

EOMG:

These patients are considered the main target for thymectomy, as they have an enlarged and hyperplastic thymus. Thymectomy should be performed early in the course of the disease in patients not experiencing full remission after administration of AChE-Is, in order to prevent the peripheral seeding of autoreactive T cells [16].

LOMG:

Thymic abnormalities are only seen in a minority of patients in this group. The patients with an enlarged and hyperplastic thymus are usually middle-aged, with disease manifestations resembling EOMG, and should probably be treated as such. Absence of antibodies to titin and RyR increases the chance for a positive effect of thymectomy [16].

Thymoma MG

All patients diagnosed with a thymoma should undergo thymectomy irrespective of MG-related symptoms. The focus of treatment is directed at removing the tumor, rather than gaining improvement of MG symptoms [65]. Surgical removal is sufficient for non-invasive thymomas [16].

Anti-MuSK MG

Thymectomy in anti-MuSK positive MG is associated with poor outcome [92], or has not shown any effect [93]. It is in most centers not recommended for patients with anti-MuSK MG.

Seronegative MG

Thymectomy as treatment of seronegative MG is controversial, and the discovery of low-affinity antibodies has made this group more heterogeneous [30]. Studies have shown an effect of thymectomy in this group to be similar to what was observed in seropositive MG [94-95]. Investigations on the effect of thymectomy in patients with low-affinity antibodies have not been performed. Seronegative MG patients with early onset and an enlarged thymus should probably be treated as EOMG patients.

Myasthenic crisis

Traditionally, myasthenic crisis (MC) is defined as an acute worsening of MG causing respiratory failure and the need for hospitalization and ventilator support. A variety of conditions, including infection, surgery, systemic illness, fever, emotional stress, pregnancy and certain drugs, can exacerbate symptoms in MG patients. At least 70% of cases of MC are caused by infections. The development and the progression of the condition can be rapid, necessitating close observation in specialized care units. [96]

Patients with MC present with respiratory stress and worsening of MG-symptoms. In patients known to have MG, the diagnosis can be readily made, whereas undiagnosed patients may require a more extensive diagnostic work up, as previously described. Pulmonary function should be assessed at least every four to six hours with peak air flow measurements and/or arterial blood gas examination. [96]

When treating MC, the primary goal is to establish an adequate mechanic respiratory support and, if necessary, to stabilize the patient via other standard intensive care unit procedures. This should precede pharmacological treatment options. The use of AChE-Is in treatment of MC have been reviewed [96]. In critically ill patients, oral absorption and drug half life may be altered and over dosage may cause increased weakness and interfere with extubation. However, no randomized, controlled trials have been done to investigate the efficacy of AChE-Is in treating MC.

Plasmapheresis and IVIG are both equally effective treatment options in worsening MG [84]. IVIG tend to be preferred because of the cost, potential side-effects of PE, and a simpler procedure. However, PE shows a slightly higher responder rate, and probably a slightly more rapid onset of action [96-97]. High-dose intravenous methylprednisolone has been suggested to be beneficial in short-term treatment of MG [61], and can be combined with either PE or IVIG [96].

After successful treatment of MC, long term immunosuppressive treatment is recommended to maintain the improvement from PE or IVIG. Prednisolone, which is gradually tapered until minimum effective dose is reached, together with AZT is usually chosen [65]. If stopped during MC treatment, the re-inctroduction of AChE-Is should follow patient remission and stabilization.

Myasthenia Gravis and the heart

Reports on cardiac abnormalities in MG patients are numerous, but no causal link has been found. Almost half of all MG patients and 97% of thymoma-MG patients have antibodies towards heart muscle antigens [98], and serum from some MG patients binds to conductive tissue in heart [37]. Antibodies targeting adrenergic β-receptors [36] and muscarinic AChRs [99] have since been identified. Myocarditis in MG patients, even in the absence of cardiac symptoms, has been demonstrated [100-105]. These cases are most often associated with thymoma MG.

Electrocardiogram (ECG) changes in MG patients have been thought to reflect cardiac conduction abnormalities possibly related to the disease. However, many types of abnormalities have been observed, and they are not MG-specific. The ECG changes include inverted T-waves and ischemic-like ST-segment abnormalities [106], QT-prolongation [107], systolic time interval abnormalities [108], terminal notching of the QRS-complex [109], right bundle branch block and arrhythmias [110]. However, not all studies performed have been able to find convincing ECG changes in MG patients [111]. Together, these observations are regarded as indicative of altered cardiac conduction in MG patients. As the changes are diverse and not specific for MG, the role of ECG in the assessment of MG-associated heart disease is uncertain.

Claims of reduced cardiac function were published in 1992. Johannessen et al. found reduced diastolic filling rate, but normal ejection fraction (EF), in a group of 25 MG patients without known heart disease [112]. Another study published a few years later observed a 40% post-exercise drop in global EF among the MG patients studied [113]. A recent study from our research group could not reproduce these observations, but found pyridostigmine-reversible reductions in atrioventricular-plane velocity and tissue Doppler peak systolic strain between MG patients and controls [114].

These reports and the knowledge of existing antibodies capable of binding to cardiac tissue, offers an array of theoretical mechanisms on how cardiac function may be affected in MG: Antibody mediated disturbances in conductivity could lead to arrhythmias, antibodies towards RyR may interfere with calcium handling in cardiomyocytes and alter cardiac inotropy, and interference with mAChRs may give rise to autonomic dysregulation with subsequent impaired cardiac function. Recognition of epitopes on heart muscle by the immune system could induce an immune mediated attack on the myocardium with complement activation, inflammatory cell infiltration and cell death as a consequence.

In several of the previously mentioned studies, the observed changes in heart function among the MG patients were reversible by pyridostigmine [108, 112, 114]. The effects of AChE-Is on cardiac function have been demonstrated through both invasive and non-invasive procedures. A single dose of 45mg pyridostigmine increased the ventricular refractory period during high heart rate in patients undergoing intracardial electrophysiological examinations [115]. However, the patients included in this study were suspected of having heart disease and used a variety of other medications. Dewland et al. examined the effect of 30mg pyridostigmine on healthy subjects, either well trained or sedentary, and found an effect on resting heart rate and post exercise heart rate recovery in the sedentary individuals only [116]. It thus seems that AChE-Is have an effect on cardiac function, but it remains unclear whether this is because AChE-Is act on the myocardium directly or through altering the autonomous nervous system. The differences between MG patients and controls in response to intake of AChE-I could also be explained by down-regulation of AChRs in MG-patients that are being treated with AChE-Is. When the drug is withdrawn before the study, its effects when re-initiated would be less than in control-subjects. Nonetheless, the observation that AChE-Is affect cardiac function in MG patients,

but not in controls, clearly suggests that there is AChE-I responsive changes in cardiac function in MG patients.

Heat Shock Proteins

The heat shock response was first described by Ritossa in the early 1960s [117]. By mistake he incubated Drosophilia at higher temperature, and observed a different chromosomal puffing pattern. He noted that this response was rapid, occurring within minutes, that a similar pattern occurred in other species, and that mitochondrial uncouplers and anaerobic conditions produced the same response [117]. Since then a various of stimuli have been shown to induce the heat shock response, including amino acid- and glucose analogs, heavy metals, protein kinase C stimulators, calcium increasing agents, ischemia, microbial infections, hormones and antibiotics, but the name heat shock persists [118]. The heat shock proteins (Hsp) are divided into subfamilies according to weight [119], and play a crucial role as chaperones in protein folding and maintaining endoplasmic reticulum homeostasis [120]. In addition, the HSPs are believed to have immunostimulatory properties, both by means of antigen presentation, but also per se.

During the last 10 years, numerous reports on the immunological role of Hsp70 and other Hsps have been published. The immunological response to Hsp70 stimulation is widespread and includes dendritic cell maturation and increased cytokine production [121], receptor mediated monocyte activation with subsequent increased expression of pro-inflammatory cytokines [122], activation of Natural Killer cells increasing their lytic capacity [119]. Hsp70 and Hsc70 (the constitutively expressed form of the protein) have been shown to chaperone immunogenic peptides into the MHC class I antigen presenting pathway [123]. In addition to being an intracellular chaperone, reports on increased efficacy in antigen presentation, possibly through extracellular chaperoning have been published [124].

One theory regarding peptide-free Hsp70 activation of the innate immune system is that Hsp70 can act as a danger signal in a situation of necrotic cell death, with release of Hsp70 to the surroundings. This implies that the immune system reacts to the dangerous situation rather than to the presence of foreign or non-self antigens [125].

However, some concern has arisen around the role of Hsp70 in immunological signaling. Hsp70 is usually acquired through genetically modifying Escherichia coli to express human

Hsp70 cDNA, and the final product may be contaminated with bacterial products, such as lipopolysaccharide (LPS) and lipoprotein. In fact, the observed Hsp70 signaling pathways, i.e. Toll like receptor 2 and -4, are the same as LPS [126]. Some studies which used Hsp70 of bacterial origin demonstrated effects of Hsp70 using nanogram quantities of the protein [122, 124], whereas Hsp70 purified from liver, had no effect even when used in 100-1000 fold greater concentration [127]. To circumvent the possibility of contamination, heat inactivation (to remove Hsp70 activity) and polymyxin-B (to inhibit LPS) treatment of the preparations have been used. These measures are by some considered inadequate, as LPS have been shown to be heat sensitive, and because contaminants other than LPS may be present [128].

The contamination issues make it difficult to interpret many of the studies, and force scientists to put more thought and effort into the process of how Hsp70 is acquired. The use of eukaryotic cells for Hsp70 expression to avoid contamination with pathogen associated molecular patterns (PAMP), such as LPS, is an alternative approach. Recombinant Hsp70 in combination with IL-2 stimulates several subsets of T-lymphocytes to produce a range of cytokines and induce proliferation of $CD4^+$ T-cells. Granzyme B mRNA levels, along with secreted amounts of protein, were significantly increased by Hsp70 in $CD3^+$, $CD4^+$ and $CD8^+$ T-cells [129]. In mice, anti-Hsp70 applied epicutaneously before treatment with an allergy inducing agent caused a systemic tolerance to the agent. The mice were able to mount an immune response to other allergy inducing agents, and the tolerance could be adoptively transferred when using spleen cells [130]. This provides both direct and indirect evidence that Hsp70 does play a role in immunoregulation, but the signaling pathways and -effects may be different from originally reported.

Involvement in disease:

Hsp70 is believed to be involved in the pathogenesis of several autoimmune disorders. Increased levels of anti-Hsp70 are observed in Behçet's disease (BD) together with increased levels of free Hsp70 [131]. This study included patients with RA and recurrent oral ulcerations in addition to healthy individuals as controls. All patient groups had increased levels of free Hsp70 compared to healthy controls, but only BD patients had increased concentrations of Hsp70-antibodies.

In patients with Grave's disease, the 8.5kb *Pst*I HSP70 allele has been shown to be associated with the disease. Furthermore it was found that, in both patients and controls, the presence of the allele was strongly associated with deletion of the C4A gene, encoding serum complement factor 4a. However, the frequency of the 8.5kb *Pst*I HSP70 allele remained higher in Grave's patients than in controls also in the absence of the C4A gene deletion, suggesting that the HSP70 locus may have immunological properties [132]. Another genetic study has also demonstrated weak association between certain HSP70 polymorphisms and Grave's disease [133].

Multiple sclerosis patients have increased levels of antibodies against Hsp70 and Hsc70 as well as DnaK (the bacterial homologue of Hsp70) when compared to patients with motor neuron disease [134]. The anti-Hsp70 and anti-Hsc70 antibodies were not specific in binding only to one of the proteins. There was only a partial overlap between the anti-Hsp70/anti-Hsc70 "antibody pool" and antibodies binding to DnaK. CSF from MS patients with high titer of antibodies was capable of inducing IL-8 and TNF-α production in vitro when administered together with Hsp70, but this did not apply for CSF from Guillain-Barré syndrome (GBS) patients, even though such sera is known to have high titers of anti-Hsp70 [134]. A higher proportion of T lymphocytes from MS patients than from healthy controls react to Hsp70 derived from Mycobacterium tuberculosis. T cell cross reactivity with human Hsp70 derived peptides was also observed [135].

In vivo studies in mice demonstrated the properties of Hsp70 as an adjuvant in inducing cytotoxic T cell mediated destruction of pancreatic islet β-cells causing diabetes [136]. Experimental autoimmune neuritis (EAN) is an animal model for studying GBS. Thirteen days post immunization with a neuritogenic peptide, there was an increase of Hsp70 positive cells in the sciatic nerves accompanied by an increase in Hsp70 mRNA in cells from the sciatic nerve and from lymph nodes [137]. Antibodies against HSP70 family members have been detected in sera from patients with sensory neuronal hearing loss [138], systemic lupus erythematosus [139], juvenile idiopathic arthritis [140] and MG [141]. These studies have focused on the presence of antibodies, and have not aimed at providing any etiological evidence.

The other study which addressed antibodies towards members of the HSP70 family in MG patients, tested for antibodies against heat shock cognate protein 71 (Hsc71) [141], also known as heat shock 70kDa protein 8, according to the HUGO Gene Nomenclature Committee. They found that the anti-Hsc71 antibody levels decreased in patients when they had improved on therapy. Therapy, however, included both immunosuppressants and plasmapheresis. By not specifying which patients received what kind of treatment, it is difficult to conclude whether the decline in antibody titers was a result of immunosuppression or plasmapheresis, or whether the antibody decline is the cause or the effect of improvement.

Matrix Metalloproteinases

The matrix metalloproteinases (MMP) is a group of 23 zinc-dependent enzymes [142-143]. They act as endopeptidases capable of cleaving a range of extracellular matrix (ECM) proteins as well as non-ECM substrates. Most cells synthesize MMPs and secrete them immediately, whereas inflammatory cells can produce for storage. Originally named based on their substrate specificity, the enzymes are now named MMP-1 through -26, with no MMP-4, -5 or -6. Most MMPs share a pro-domain and a catalytic domain, where the catalytic domain contains the zinc binding site [144]. The MMP-group consists of both membrane bound and secreted proteins [143].

The MMPs both as a group and as individual enzymes have an array of potential substrates, including ECM components (i.e. collagens, elastin, fibronectin, laminin), coagulation factors (i.e. fibrin, fibrinogen), immunoactive proteins (i.e. IL-1β, IL-8, pro-TNF, complement factors) and other MMPs [142-143]. The wide substrate specificity has potential deleterious effects if not properly regulated. The secreted MMPs are often bound to proteins on the cell membrane, limiting their area of impact to the pericellular space [143]. It has been shown that cells are able to determine the localization of different substrates through binding to surface receptors, and that the secretion of MMPs is directed to the area where substrate-receptor binding occurred [145].

Expression of MMPs is normally low in tissues, but increases during conditions requiring remodeling of the ECM [146]. Main, but not sole, regulation of MMP gene expression occurs at the Activator Protein-1 (AP-1) binding site, found in the promoter region of the inducible

MMP genes [147]. AP-1 complexes are heterodimers of members of the protein families *jun* and *fos*. Co-transfection of *jun* and *fos* enhances MMP-1 expression, and *c-fos* is required for induction of mouse MMP-3 and MMP-13 gene expression [143]. The promoter sequence of the inducible MMP genes also contains polyoma enhancer A binding protein-3 (PEA3) elements that bind ETS transcription factors, which are believed to be coactivators when forming complexes with factors binding to AP-1 [146]. Several stimuli converge onto these regulatory elements through intracellular kinases: Growth factors and mitogens activate the ERK1,2 pathways, oxidative and osmotic stress stimulates the ERK5,6 pathways, and the jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is stimulated by environmental stress and inflammatory cytokines [143]. Altough primarily regulated on the transcriptional level, regulation downstream of transcription, such as modulation of MMP mRNA stability, has been reported [148].

MMPs are secreted in a pro-form, requiring activation, often through catalytic cleaving, in order to exert its function. Activation is achieved through various enzymes, such as plasmin, urokinase plasminogen activator and tissue-type plasminogen activator, but also through proteolysis by other MMPs. The biochemical reason for this mode of activation relies in the blocking of the catalytic site by a cysteine residue (Cys^{73}) in the pro-domain of the enzyme. This Cys^{73} is common for most MMPs. Dissociation of the cysteine residue from the zinc atom in the active site, either through protein denaturation (e.g. by use of SDS or organomercurials) or through proteolytic cleaving (by other proteases or auto-proteolysis), activates the MMP. This process is called Cysteine Switch Mechanism [143].

Two types of ambient inhibitors of the MMPs exist, the four variants of tissue inhibitors of metalloproteinases (TIMP1-4) and the inhibitors of metalloproteinases (IMPs). These factors inhibit all types of MMPs by binding to the active site of the enzyme. Although all are broad spectrum MMP inhibitors, some differences exist in terms of MMP-specificity, mechanism of action and ability to inhibit other types of proteinases. The MMPs are also inhibited by the general proteinase inhibitors α2-macroglobulins [149].

Involvement in disease:

In RA, MMP3 is increased in both serum and synovial fluid compared to the levels in patients suffering from degenerative osteoarthritis. The elevated serum levels correlated

with degree of MMP3-immunostaining and immune cell infiltration in synovial tissues, suggesting that the serum level increase was due to local production in inflamed joint tissues. Knee replacement surgery caused a decline in MMP3 levels, which were maintained for up to 4 weeks, further supporting the local production theory [150]. In addition to MMP3, MMP2 and MMP9 serum levels and enzymatic activity are increased in RA patients [151]. The increased MMP3 levels in RA was confirmed in another study where RA patients were used as controls when examining serum levels of MMP3 in patients with systemic lupus erythematosus (SLE), in particular SLE patients with active renal disease with biochemical signs of renal involvement (persistent proteinurea, cellular casts, decreased creatinin clearance, and serum uric acid levels) [152]. MMP2 and -9 levels and activity are also increased in SLE patiens [151]. Matache et al. found increased levels of spontaneously released MMP9 from peripheral blood mononuclear cells (PBMCs) but unchanged levels of secreted TIMP-1 from patients with SLE, providing a potential source for the increased MMP9 levels [153].

Both the concentration and enzymatic activity of MMP9 is increased compared to healthy controls in saliva and in biopsies from the salivary glands of Sjögren's Syndrome (SS) patients. When comparing biopsies from healthy controls and the SS patients, there was no difference in expression patterns of either MMP9 or MMP2. However, some neutrophils showed intense MMP9 staining in the SS group only [154]. Patients with primary SS with severe sialadenitis had increased local MMP3 expression in minor salivary glands compared to patients with secondary SS [155]. Salivary gland expression of MMP2, -3 and -9 together with TIMP-1 and -2 have also been assessed by means of RT-PCR and western blotting from salivary gland biopsies, and showed MMP3/TIMP-1 and MMP9/TIMP-1 ratios above 1, with the latter being the highest. Acinar changes seemed to correlate with increased enzyme/inhibitor-ratio, suggesting that MMP9 accounts for the main proteolytic activity of the enzymes measured [156].

Several of the MMPs have been thoroughly studied in patients with MS and in MS models, mainly experimental autoimmune encephalitis (EAE). CSF samples from MS patients have increased levels of MMP2 and -9 [157-159], and MMP9 serum expression correlates with disease activity measured by means of MRI and clinical evaluation [159-160]. The MMP9 increase has been shown to precede the appearance of new gadolinium enhanced lesions

on MRI [161]. This, in addition to the observation of MMP2 and -9 expressing macrophages and lymphocytes around perivascular cuffs [162], is fueling the theory that MMP9 is important in immune cell migration across the blood brain barrier. Support for the role of MMP9 in MS development comes from animal studies in which young MMP9 null mice were less susceptible to develop EAE than wild type controls. A compensatory increase in MMP9 was observed in MMP2 null mice which developed a more severe disease with earlier onset than controls, whereas MMP2 and -9 double null mice failed to develop any disease at all [162].

MMP3 levels are increased in serum from patients both with seronegative and seropositive MG. In mice with experimentally induced MG through immunization with a synthetic peptide from the human AChR, treatment with a dual altered peptide ligand caused decreased T cell mediated MMP9 activity [163].

In experimental autoimmune neuritis (EAN), an animal Guillain–Barré syndrome model, MMP2 and -9 mediated breakdown of β-dystroglycan has been shown to contribute to the development of the disease. The α/β-dystroglycan complex harbors an important role in myelin formation and stability, and disruption of this complex can lead to peripheral nervous system dysmyelination. Increased levels of MMP2 and -9 were found, using western blotting and immunohistochemistry, in sciatic nerves of EAN rats compared to control animals. Using captopril as an MMP inhibitor from the time of disease induction, the captopril-treated animals developed a milder disease phenotype with less demyelination, less β-dystroglycan cleavage, less MMP2 and -9 expression and reduced sciatic nerve inflammatory cell infiltration [164].

This review shows that MMPs play an important role in autoimmune diseases. Judging from the variety of different immunological diseases with increased levels of the enzymes, MMP effects are not disease specific.

Guillain-Barré syndrome

Guillain-Barré syndrome (GBS) is an autoimmune peripheral nervous system disorder. It consists of at least four subtypes, with the acute inflammatory demyelinating polyradiculoneuropathy (AIDP) type being the most common. The disease occurs after an infection with campylobacter jejuni in 25% of cases, due to similar peptide sequences

occurring in the peripheral nervous system and in the bacterial wall. In AIDP it is believed that the myelin breakdown occurs either through T-cell mediated activation of macrophages or due to antibody-binding and complement mediated attack on the Schwann cells. Axonal damage is not a dominating feature, but can occur as a secondary phenomenon if the demyelination is severe. In acute motor axonal neuropathy (AMAN), and in acute motor and sensory axonal neuropathy (AMSAN), the macrophages are activated through Fc mediated binding of antibodies targeting antigens on the axonal membrane, leaving the myelin sheaths intact.

The initial symptoms are typically pain, numbness, paraesthesias or weakness in the limbs, most often starting distally with proximal spreading as the disease progresses. Autonomic involvement is common and may give rise to symptoms such as hypertension, urine retention, ileus, sinus tachycardia and arrhythmias. Progression of the weakness can cause respiratory difficulties. The deteriorating phase of the acute disease lasts for 2-3 weeks, after which the patient enters a plateau phase and then begins to recover. The prognosis for AIDP is generally good, but some patients will not experience a full recovery [165].

Treatment includes plasmapheresis and intravenous immunoglobulins [166]. Corticosteroids are considered ineffective for AIDP [167].

AIMS OF THE STUDY

1. To assess the potential cytotoxic properties of MG sera in vitro on various cell types. (papers I and II)

2. To measure potential non-antibody, disease mediating factors in MG (papers III and IV)

MATERIALS AND METHODS

Patients and controls

The MG diagnosis was made based on the clinical presentation of symptoms, presence of AChR antibodies in serum and electrophysiological findings (presence of >10% decrement of the compound action potential using repetitive stimulation at 2-3 Hz, and increased jitter using single fiber electromyography). Patients included in paper I and II were diagnosed at the Department of Neurology, Haukeland University Hospital, Bergen, Norway whereas the patients in paper III and IV were diagnosed with MG at Moorfields Eye Hospital, Neuro-ophtalmology Clinic, London, UK, and followed by Gordon Plant (co-author on papers III and IV).

Control sera included Norwegian blood donors (for papers I – IV), Norwegian GBS patients and Norwegian MS patients (paper III and IV). The GBS and MS patients were all diagnosed and followed by physicians at Department of Neurology, Haukeland University Hospital. The MS diagnosis was made based on the Poser criteria [168]. GBS patients were diagnosed according to Asbury and Cornblath's clinical criteria [169].

All patient samples used in the experiments reported were collected and stored as 1-2 ml aliquots below -20°C.

Cell culture

All cell culture experiments were performed in sterile conditions using properly maintained laminar air flow benches. For incubation of cells water jacketed incubators set to 37°C with 5% CO2 / 95% O2 were used. Culturing media was stored and supplemented when needed according to the manufacturer, and heated to 37°C using a water bath before application.

Cell viability assays (paper II)

Cell viability was quantitatively measured using two different methods:

Adenylate Kinase (AK) release assay [170]

The enzyme AK is released upon the loss of cell plasma membrane integrity when cells are cultured in vitro. The amount of AK released into the growth medium will reflect the degree of cytotoxicity. The kit used (ToxiLight®, Cambrex (previously Lonza)) relied on a two step

process for detecting the amount of AK released. The first step is catalyzed by AK and provides ATP for assay bioluminescence, whereas the second step is catalyzed by Luciferase and produces light from ATP and luciferin.

$Mg^{++}ADP + ADP \leftrightarrow Mg^{++}ATP + AMP$

$ATP + Luciferin + O_2 \rightarrow Oxyluciferin + AMP + PP_i + CO_2 + Mg^{++} + LIGHT$

The emitted light intensity is related linearly to the concentration of AK in the medium, and can be measured on a luminometer.

Annexin-V-Fluos kit [171]

This assay uses Annexin-V-FITC and Propidium Iodide (PI) to stain the cells before they are examined using a flow cytometer. When a cell undergoes apoptosis, phosphatidylserine (PS) is translocated from the inner to the outer side of the plasma membrane, while the plasma membrane integrity remains intact. Annexin-V is a Ca^{2+} dependent phospholipid binding proteins with high affinity for PS, thus making it suitable for labeling cells undergoing apoptosis when conjugated to a fluorescent dye. The flipping of PS to the outer membrane also occurs during necrosis, but contrary to apoptosis, the plasma membrane does not remain intact. To differentiate apoptotic from necrotic cells, PI is used to stain DNA, as it will not penetrate an intact plasma membrane [172]. After labeling the cells according to assay instructions [171], the cells (10.000 readings per sample) were examined on a BD FACS Aria SORP flow cytometer (BD Biosciences, San Jose, CA, USA) using 488 and 532 nm lasers with 525/50 and 610/20 bandpass filters, respectively. Treatment of the cells with 0.01 M H_2O_2 for 24 hours served as a positive control, from which the appropriate gating was performed.

Antibody assays (papers III and IV)

In paper III, anti-Hsp70 antibody total (IgG, IgM and IgA) concentrations were measured using anti-Human Hsp70 ELISA kit (Stressgen, MI, USA). Preparation of serum samples and incubation times were performed according to assay instructions. According to the information given by the manufacturer, the sensitivity of the assay was 6.79 ng/ml. Both the inter- and intra-assay coefficient of variation was <10%. All readings were within the standard curve.

For the measurements of MMP2, -3 and -9 levels in paper IV, Quantikine Human Total MMP2, -3 and -9 ELISA Kits (R&D Systems, Minneapolis, MN, USA) were used according to assay instructions. Sensitivity (mean minimum detectable concentration) and intra- and inter-assay coefficient of variation for the assays were as stated in Table 1. All readings were within the standard curve.

		Coefficient of variation	
	Sensitivity (ng/ml)	Intra-assay	Inter-assay
MMP2	0,16	4,80 %	7,70 %
MMP3	0,009	6,10 %	7,80 %
MMP9	<0,156	2,30 %	7,50 %

Table 1: Precision of ELISA assays used in paper IV. Numbers are supplied by the manufacturer.

The optical density for all assays was measured at 450nm with 590nm correction using Thermo Multiskan EX plate reader.

In vitro live cell calcium measurements

The results from these experiments are not included in the four papers for which this thesis is based upon. This project was partly a continuation of the work performed by Skeie et al., where he showed that anti-RyR antibodies can inhibit binding of ryanodine to the receptor [41], and this can inhibit calcium release from terminal cisternae fractions [173]. Finally, MG patients with antibodies towards RyR have a more severe MG, than the anti-RyR negative MG patients [174]. The possibility of a pathogenic role for the RyR antibody in MG, led us to trying to establish a model for measuring the effects of the antibody on calcium release in live muscle cells.

Skeletal muscle cells (human skeletal muscle myoblasts (HSMM) (Lonza, Switzerland) and C2C12 muscle myoblasts (Sigma-Aldrich, Norway)) and were seeded onto No. 0 35 mm glass bottom culture dishes (MatTek Corporation, Ashland, MA, USA), grown to confluency and differentiated for 3-7 days. Differentiation media contained DMEM supplemented with

2mM L-glutamine and 2% horse serum. After differentiation, the cells were maintained in appropriate growth media for 3-5 days. Immediately before the experiments both cell types were loaded with 1-2µg/ml Fura-2-acetoxymethyl ester (Invitrogen) at room temperature for 60-120 min before a 30-60 min de-esterification period at 37°C.

The glass bottom dishes were fitted in a custom made, heated perfusion chamber frame on the stage of an Olympus IX-70 with a x40 UAPO objective. A gravity driven perfusion system passing through a Warner TC344-B perfusion inline heater fed the perfusion chamber at 2 ml/min while maintaining the chamber temperature at 36-37°C. Switching between fluids was done automatically with magnetic valves. Regions of interest (ROI) were defined using the image analysis software and drawn along the borders of cells displaying an elongated, multinucleated phenotype. Background fluorescence was measured in areas adjacent to the cell ROIs and subtracted from the recordings.

Alternating excitation with 340 nm and 380 nm wavelengths was performed using a dual-excitation wavelengths system (Delta-Ram) from Photon Technologies (PTI). The signal passed through a 510 nm low pass filter before being recorded by an IC-200 intensified CCD camera with at a frequency of 1,6Hz. Image Master (PTI) software was used to calculate the ratio between the 510nm signals obtained from excitation with 340 and 380nm wavelengths using the following formula:

$$\boldsymbol{Ratio} = \frac{\boldsymbol{340nm\ cell - 340nm\ background}}{\boldsymbol{380nm\ cell - 380nm\ background}}$$

The cells were perfused with perfusion buffer adjusted to pH 7.4 containing 137mM NaCl, 5.4mM KCl, 0.5mM MgCl2, 1.8mM CaCl2, 11.8mM HEPES-NaOH and 1g/l glucose for at least 3 minutes until a one minute stable baseline ratio was obtained. Caffeine (Sigma-Aldrich, Norway) was dissolved in perfusion buffer to a final concentration of 0.1, 1 and 10mM to stimulate calcium release from the cells [175-177]. Dantrolene 5µM was included as a negative control.

Statistical methods

Differences between patients and controls in paper II were assessed using one-way analysis of variance (ANOVA).

Normality assessment for continuous variables in papers III and IV was performed using the Anderson-Darling test.

The choice of a statistical test for group comparison in papers III and IV depended on the outcome from the normality assessment. If normality was present, continuous variables were compared using a two-sample t-test. In cases of non-normality distribution, the Mann-Whitney U test was employed.

A confidence interval of 95% was used as significance level in all experiments.

SUMMARY OF RESULTS

Morphological and cytotoxic effects of Myasthenia Gravis patient sera on human cells in culture (papers I and II)

Of the 13 MG patient sera tested on primary human myotubes (paper I), 2 sera (denoted *a* and *b*) were able to induce dramatic morphological changes in the muscle cells. The myotubes transformed from an organized elongated phenotype to appearing in a disorganized, contracted and irregular pattern. In addition, perinuclear inclusion bodies appeared. The factors responsible for these changes had molecular weight above 100kDa. Sera a and b also caused a decrease in cell number when incubated over a period of three days. As myotubes are multinuclear, the number of DAPI-stained nuclei was used to assess cell number. Serum from a third MG patient, where serum was drawn during a MG crisis, just as for serum a and b, also caused morphological changes and formation of inclusion bodies, but to a lesser degree. Nine of the remaining 10 patient sera caused a varying degree of morphological changes, ranging from mild to moderate, and a lesser degree of inclusion body formation than what was observed in sera a – c. However, they were all different from controls. Cells treated with serum from one MG patient (m) were indistinguishable from controls.

Serum b caused membrane blebbing in the myotubes. DAPI-staining following treatment with MG sera revealed intranuclear vesicle-like structures, which could not be seen in the nuclei of cells treated with control serum. The sera which caused the most marked morphological changes using CV staining also led to a disorganization of the actin cytoskeleton, with the actin fibers running in multiple directions. The less potent MG sera were able to induce some disturbances in the actin organization, but not to the same extent as sera a and b.

The changes observed were dose- and time dependant. The formation of inclusion bodies occurred after 24 hours, after which retraction of the cell membrane, formation of discrete multicellular clusters of cells and a progressive loss of cell number over 4 days occured. Sera diluted 1:10 were more potent in inducing cellular changes than sera diluted 1:100.

Heat-inactivation of the sera did not inhibit the observed changes and indicates a non-complement mediated mode of action.

Given the results in paper I and the abundance of reports suggesting a link between cardiac disease and MG, we expected to observe a morphological and/or cytotoxic effect of human MG sera on cultured human heart cells. However, none of the 15 MG sera included in our experiments caused morphological changes. No differences in cell number after incubation with sera from patients and controls could be seen. The relative level of AK released from cells treated with MG sera did not differ from healthy human control sera. Assessment of cell death and apoptosis induction by MG sera was performed by Annexin-V- and PI-staining and flow cytometry. Control sera induced cell death in 8.4% of the cells, measured as percentage of cells positive for PI-staining. This did not differ from the 9.3% PI-positive cells after treatment with MG sera (p=0.694). Less than 1% Annexin-V positive cells could be detected in both patients and healthy controls, indicating no apoptosis activation (Figure 1).

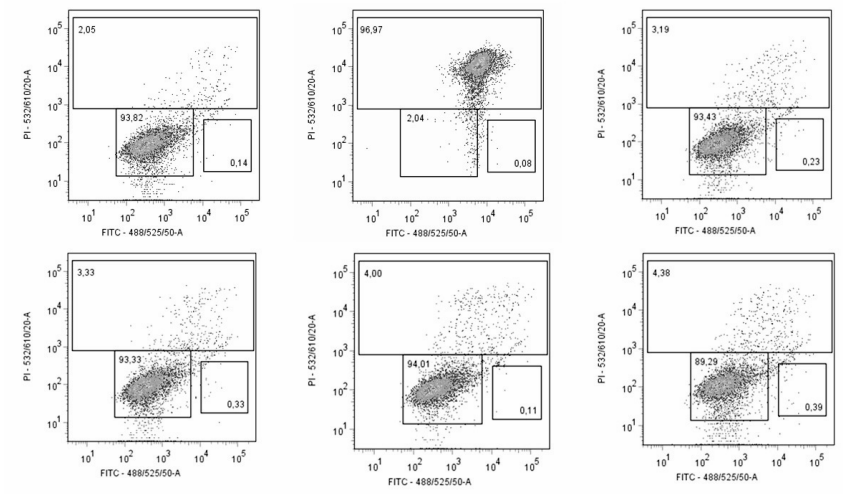


Figure 1: Dot plots of Girardi cells stained with Annexin-V-FITC and PI after 24 hours treatment with human control sera (a), 0,01M H_2O_2 (b) and sera from 4 different MG patients (1 seronegative (c) and 3 seropositive (d-f)). Double negative cells are regarded as live, whereas PI positive cells are counted as dead cells. The selected patient plots are representative for all MG sera tested.

The results in paper II were not dependant on serum incubation time or concentration.

Potential non-antibody disease mediating factors in MG (papers III and IV)

Sera from 129 MG patients were used for both paper III and IV. The MG patients included were categorized depending on their symptoms being restricted to the ocular and periocular muscles (ocular MG (OMG)) or affecting other muscles (generalized MG (GMG)). However, all MG patients had involvement of the ocular or periocular muscles, and in cases of generalized disease, the symptoms were mild. This is further discussed on page 50. The OMG and GMG groups were similar with regard to age, sex and presence of anti-AChR antibodies. Of the 94 GMG patients 36 had detectable levels of anti-AChR antibodies, 53 were negative and 5 unknown. The OMG group had 16 anti-AChR positive, 18 negative and 1 unknown. In paper III, sera from 37 patients with untreated GBS and 49 with untreated MS were included as disease controls. 41 blood donor sera were used as healthy controls in both papers.

Both GMG and OMG patients had higher anti-Hsp70 levels than healthy controls, with median values being 271.0, 333.0 and 171.9 µg/ml, respectively, ($p<0.001$). They were also significantly higher than in the MS patients ($p=0.0004$). Median anti-Hsp70 concentration in GBS patients was 471.8 µg/ml, this being significantly higher than in all the other groups (Figure 2).

No correlation between patients' age at time of inclusion in the study and anti-Hsp70 levels was found. As all patients included in the study had received their MG diagnosis within the last two years before serum was drawn, age at inclusion was used to categorize patients into either EOMG or LOMG.

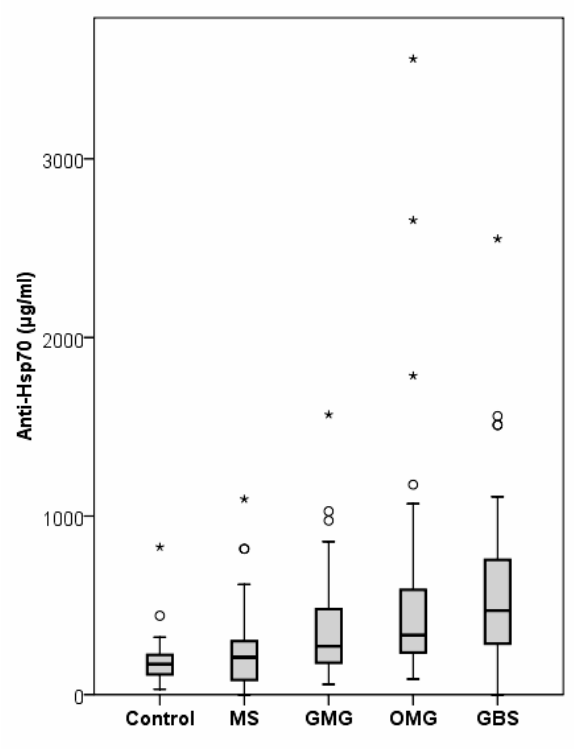


*Figure 2: Box plot illustrating the anti-Hsp70 antibody concentrations in OMG and GMG patients and controls. The boxes represent interquartile range (IQR). Outliers within 1.5 IQR of the boxes are marked with whiskers, whereas outliers between 1.5 and 3 IQR, and 3 IQR of the boxes are denoted **O** and *, respectively.*

When examining MMP levels in the MG patients, no difference between the OMG and GMG group or between the anti-AChR positive or negative patients was observed for either MMP2, -3 or -9. When comparing all the MG patients to controls, there were significantly higher concentrations in the patient group for MMP2 and -9 (Figure 3 and Table 2). The same difference could not be found for MMP3. It was noted that female MG patients had significantly lower MMP3 levels than the male MG patients (p=0.0002) and controls (p=0.0016). It was also noted that a subgroup of 19 patients had MMP3 values exceeding the maximum concentration measured for any of the controls, and by a factor of 1.4 – 2.7.

This MMP3-High group contained more male patients (15 vs. 4; p=0.01). This group did not differ with regard to MMP2- (p=0.95) or MMP9-levels (p=0.91), anti-AChR-status (p=0.33), age (p=0.62) or MG subtype (GMG vs. OMG, p=0.59) (Table 3).

MMP2 concentration showed a significant positive association with increasing patient age (p=0.027). This age-dependence was not found for the other MMPs (MMP3: p=0.82; MMP9: p=0.25).

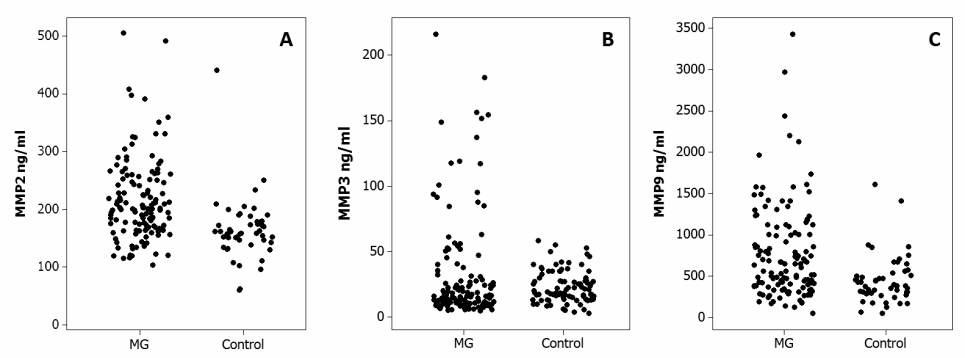


Figure 3: Scatterplot of MMP-levels in MG patients and controls. A: MMP2, B: MMP3, C: MMP9

		MG Median (IQR)	Controls Median (IQR)	Significance
MMP2	AChR-pos	207.7 (95.8)	159.7 (33.7)	p<0.001
	AChR-neg	198.7 (75.4)		p<0.001
MMP3	AChR-pos	17.6 (30.3)	20.5 (17.1)	p=0.727
	AChR-neg	16.7 (18.4)		p=0.252
MMP9	AChR-pos	495.0 (816.3)	386.4 (252.5)	p=0.001
	AChR-neg	671.5 (723.5)		p<0.001

Table 2: Median MMP-values with interquartile range in the 52 anti-AChR-positive and the 71 anti-AChR-negative MG patients. Significance is compared to blood donor controls.

	n	MMP2 (median)	MMP9 (median)	GMG/OMG	AChR pos/neg	Sex m/f	Age (mean)
MMP3-High	19	198.8	665	15/4	10/9	15/4	59.4
MMP3-Normal	110	201.3	614.5	79/31	42/62 (6)	51/59	56.7
		*NS**	*NS**	*NS†*	*NS†*	*p=0.01†*	*NS‡*

*Table 3: 19 MG patients with MMP3-values exceeding the highest value in the control group (MMP3-High) compared to the rest of the MG patients (MMP3-Normal). (*Mann-Whitney U-test, †Fisher's exact test, ‡2-sample t-test, NS: Not significant)*

In vitro live cell calcium measurements

Both HSMMs and C2C12 cells responded to caffeine stimulation with an increase in intracellular calcium levels. The effects appeared within seconds, and were dependent on the caffeine concentration used. Dantrolene inhibited caffeine induced calcium release effectively (Figure 4). Initially, human skeletal muscle myoblasts were used for the experiments. The responses obtained from caffeine stimulation were generally good, but the 340/380nm baseline ratio showed a high degree of variation between experiments. Also, these cells had a finite life span, and the caffeine responses seemed to decay when they were passaged more than 5 times. This rendered them unsuitable for further experiments.

C2C12 cells are derived from mouse, and reports of caffeine induced calcium release in these cells have been published [177]. These cells were obtained from European collection of cell cultures through Sigma-Aldrich, ensuring their genuinity. Contrary to the HSMMs, C2C12 cells are a stable cell line. The caffeine response did not decay throughout passaging, as was observed with the HSMMs. However, the variations from one experiment to the other showed a high degree of variation. These variations occurred in cells treated similarly through passaging, differentiation, loading of FURA-dye and perfusion. Changes to the differentiation protocol; either through altering the differentiation time, changing the degree of cell confluency before differentiation, or prolonging the period of time the cells were maintained in growth media before starting the experiments did not improve the

results. Modifying the FURA loading protocol both with regard to incubation time and incubation temperature caused no decreased variation.

Because we were not able to establish a stable system for measuring the changing calcium levels in live cells, the project was discontinued.

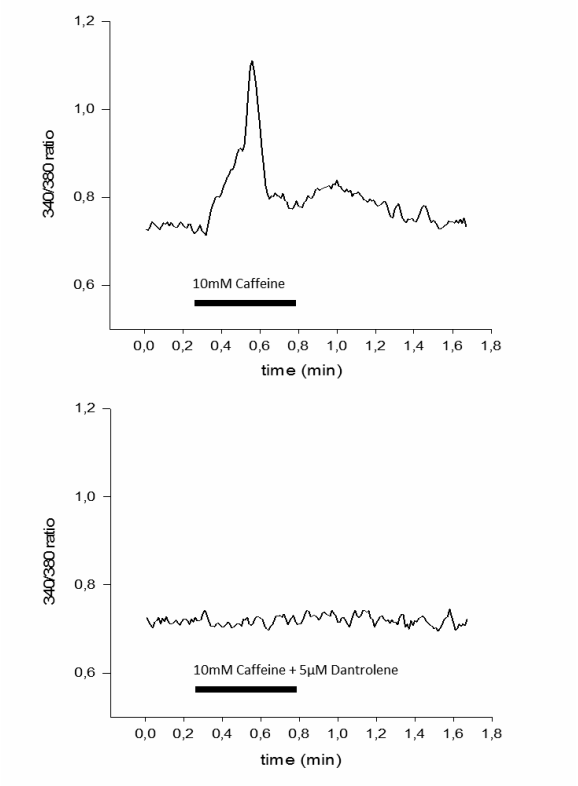


Figure 4: Graph showing the ratio between 340nm and 380nm signals from 510nm excited FURA-stained human skeletal muscle myoblasts and the response to 10mM caffeine alone or in combination with 5µM Dantrolene.

GENERAL DISCUSSION

The observation that some MG sera are cytotoxic to cultured human myoblasts indicates that serum factors in MG patients are capable of damaging muscle cells even though they lack a functioning neuromuscular synapse. Cultured skeletal muscle cells need neural stimuli to express the punctuate AChR expression pattern. This can be achieved either through co-culturing skeletal muscle cells with cells originating from the spinal cord [178], or treating the muscle cells with agrin [179]. The mode of action was not complement mediated, as heat inactivation of sera had no effect, and the factors responsible for the observed changes had a MW >100 kDa. The separation of serum according to MW was achieved by means of centrifugal filtration through a Vivaspin filtration column with a 100 kDa membrane. This fraction includes all subclasses of antibodies, but does not contain most of interleukins and potential medications present in the patients' sera.

Sera from patients undergoing myasthenic crisis elicited the greatest effect in inducing morphological changes and reducing cell number, both compared to non-crisis MG patients and controls. During plasmapheresis the patients receives anticoagulant (heparin administered according to patients weight was used). Heparin (20 IU/ml medium) was therefore included as a control when treating the cells with plasmapheresis filtrate from crisis patients. Plasmapheresis serum from a patient with small cell lung cancer and limbic encephalitis with anti-Hu antibodies was used as a control, and had no effect.

There was no apparent correlation between the anti-AChR status of the patients and the observed changes. A radioimmune assay was used to detect binding (as opposed to blocking) antibodies targeting the AChR [54]. Leite et al. published in 2008 results showing that 25 of 38 patients previously believed to be seronegative, in fact had low-affinity antibodies to the AChR. These antibodies could only be detected when subunits of the AChR were transfected and overexpressed in cells, to which the antibodies would bind [30]. The antibodies were mainly IgG1 subclass and could activate complement. Thus it is likely that a portion of the seronegative MG patients included in the studies in this thesis belong to the seropositive group, and this may have an impact on the conclusions drawn.

In the UK MG patients, we noted that 45.7% of the OMG patients had detectable levels of anti-AChR antibodies. This is as expected for OMG [13]. In the GMG group, we found only

38.3% anti-AChR positive patients, which is much lower than the 80-90% commonly reported [9]. A reason for this may be that the patients included were recruited for the studies from a neuro-ophtalmology unit to which they were referred from other physicians due to weakness primary in the ocular and/or periocular muscles. Where there were generalized symptoms, they tended to be mild. We could find no differences between the GMG and OMG patients either with regards to anti-Hsp70 antibodies or MMP-levels. It is tempting to speculate that the GMG patients, in spite of having symptoms affecting muscles outside the ocular/periocular system, should be regarded as OMG patients.

Antibodies against titin and RyR did not seem to play a major role for the cell culture response, as the three most potent sera were negative for either, although several of the sera with milder effects had anti-titin, and some also anti-RyR antibodies, although the present experiments made a major role less likely. One cannot rule out the possibility of these antibodies contributing to our observations or to the pathogenesis of MG. RyR has an important function in both skeletal and cardiac muscle in through Ca^{2+}-release from the SR, regulating excitation-contraction coupling. Disturbances in the function of this protein can give rise to disease in both skeletal muscle, such as malignant hyperthermia [180], and cardiac arrhythmias [181]. Its involvement in MG is highlighted, beyond the presence of the antibodies per se, by the fact that MG patients with anti-RyR antibodies capable of inhibit ryanodine binding have a more severe disease than those with anti-RyR antibodies not inhibiting binding of ryanodine [41]. An argument against its role in pathogenesis is that the antigen resides intracellularly, and is not available for antibody-binding. Several papers are published on the topic of antibody internalization, but most of them have in common that the antibody in question binds to an extracellular antigen before being internalized [182-183]. Internalization of antibodies against intracellular antigens has been reported [46, 184], and we cannot rule out the possibility that similar mechanisms take place in the course of MG.

The possibility of anti-RyR antibodies playing a pathogenic role in MG led us to trying to establish a model system for measuring the potential effect of these antibodies on calcium handling in muscle cells . FURA-2 was used to detect intracellular calcium levels. This is a ratiometric fluorescent dye which binds to free calcium. The calculated ratio of fluorescence emitted when FURA-2 is excited with 340 and 380nm gives a relative calcium concentration.

Two different muscle cell lines were used; one human and one murine. Stimulation of the cells with caffeine gave an increase in intracellular calcium, and the effect was effectively inhibited with Dantrolene. However, the caffeine responses varied significantly between experiments, and we were not able to obtain reproducible and predictable baseline recordings. Multiple adjustments were applied to the cell differentiation protocol, growth medium, FURA-2 loading protocol and perfusion protocol, but to no avail. The integrity of the mechanical setup was ensured by others using the same equipment and getting stable readings. These experiments have not been published.

The UK MG patients were categorized as early or late onset MG patients according to their age at inclusion in the study. All patients included were diagnosed with MG within the last two years before they were included in the study, but exact age at time of diagnosis or of symptom debut was not obtained. Hence it is possible that a patient diagnosed at age of 49 is categorized as a late onset patient if the inclusion in the study occurred approximately two years later. However, only three patients were included in the study when aged between 50 and 52 years. A comparison between the groups after excluding these three patients yielded no significant change with regard to anti-Hsp70 antibody levels ($p=0.787$) and MMP9 ($p=0.665$). For MMP2 and MMP3 a significant difference in concentrations between EOMG and LOMG patients is dependent on whether these three patients are categorized as early or late onset. Because of this uncertainty, we found that this result could not be presented as a conclusive finding.

MG patients were found to have increased levels of MMP2, MMP9 and anti-Hsp70 antibodies compared to healthy controls. We measured the concentrations only once in each patient. This limits the conclusions that can be drawn. It is not possible to conclude whether these parameters are correlated to disease severity, as we could not monitor the serum levels during the fluctuations of the disease. Clinical grading of patients' symptoms was not obtained. Levels of MMPs and anti-Hsp70 antibodies are found increased in other diseases, including, but not restricted to, autoimmune diseases [185-189]. This indicates that they are not disease specific, but their increase in several diseases affecting various organ systems suggests a role in the pathogenesis. The increased serum markers may well be due to local tissue destruction with local upregulation of MMPs and increased exposure to Hsp70 with subsequent antibody production. As many of the MMPs are important in

facilitating cell migration, any situation requiring the recruitment of certain cell types should in theory cause increased MMP-levels.

The MMP levels measured did not take into account the enzymatic activity of these proteins, just their concentration. The MMPs have wide substrate specificity and thus requires potent regulatory mechanisms. Measuring the TIMP-levels in patient sera as well as the MMP concentrations would give a more comprehensive picture and possibly have impact on the conclusions drawn. A different approach to enzymatic activity for a functional assessment is zymography. This method uses many of the same principles as sodium dodecyl sulfate polyacrylamide gel electrophoresis, also known as SDS-PAGE. The two techniques differ in that the samples are not boiled or denatured beyond the addition of SDS, the gel used includes enzyme substrate, and following electrophoresis, the gels are incubated in appropriate buffer to allow the enzymes to degrade the substrate. After incubation, the gels are stained, and areas of enzymatic digestion will appear [190]. The relationship between MMP2 and -9 serum concentrations assessed by ELISA and enzyme activity by gelatin zymography, showed that the increased serum levels of the proteins in patients with SLE, RA and type-1 diabetes mellitus corresponded with increased enzymatic activity [151, 191]. It is therefore likely that the increased MMP serum levels presented in paper IV reflect increased enzymatic activity.

As to which mechanisms MMPs may interfere with to contribute to the pathogenesis of MG, the lack of data makes this a speculation. But some indirect evidence exists. Zamecnik et al. reported in 2007 that 18 of 179 MG patients had lymphocytic infiltrations in muscle by $CD8^+$ T cells. All MG patients with such infiltrations had lymphocyte-rich thymomas [192]. A previous study which included 30 MG patients, also found lymphocytic infiltrations in skeletal muscle, but concluded that the observations were more likely to reflect disturbed immune regulation in MG, than having of causal role in the disease [193]. The capacity of MMPs to promote immune cell migration has been shown in several in vivo studies [194-196], and they are likely to be involved for the lymphocytes to extravasate into the muscle.

Of the 129 MG patients included for MMP-measurements, 19 had concentrations exceeding the highest occurring healthy controls. The whole patient group did not differ from the control group with respect to MMP3, contrary to what has been shown in a previous study

[197]. The diverging results may be due to the inclusion of patients with predominantly periocular symptoms in paper IV. Any generalized symptoms tended to be mild. The role of MMP3 in development and maintenance of the NMJ has been reviewed by Werle in 2008 [198]. Agrin is a known substrate for MMP3, and is essential for the formation of the NMJ. MMP3 null mice have increased postsynaptic AChR areas and postsynaptic membrane folds, and agrin, AChR and junctional folds are commonly seen in absence of a nerve terminal. It thus seems that the mutant mice without MMP3 lack the ability to remove unneeded agrin. MMP3 activity in the NMJ decreases with impaired synaptic transmission; whereas immunostaining shows that MMP3 is still present, suggesting the action of an inhibitor. Although the evidence seems compelling, transferring these animal- and in vitro studies to humans has to be done with caution. One hypothesis for the action of MMP3 in MG is involvement in remodeling of the NMJ following the complement mediated attack after antibodies bind to the AChRs. An increase in MMP3 levels and activity could cause an excessive breakdown of agrin resulting in an incomplete rebuild of the NMJ, as seen in MG [9]. The theory has its weakness in that a decrement in synaptic activity, as seen in MG, should, according to studies from animals [199], give reduced MMP3 activity and subsequently increased local agrin levels.

Increased circulating levels of antibodies towards Hsp70 in MG and GBS patients suggest either current or previous exposure to the antigen. Hsp70 can act both as a peptide chaperone and as a maturation signal for dendritic cells (DC) [200]. Production of intracellular Hsp70 as well as Hsp70 release to the extracellular space increase in stressful conditions [119]. Skeletal muscle cells upregulate Hsp70 transcription and translation upon depolarization [201], ischemia [202], heat stress [203], and energy and milieu challenges [204]. Disturbances in Hsp70 function and signaling by increased levels of antibodies targeting the protein may reduce the muscle cell's ability to cope with cellular stress and alter the immune response. In the development of MG, the NMJ is destroyed by antibody cross binding, complement activation and formation of membrane attack complexes [9]. The muscle cells would most likely respond to this stressor by up-regulating Hsp70 production, and the released Hsp70 can then act as a stimulatory signal to the immune system through CD14, CD40, CD91 and members of the Toll like receptor family on antigen presenting cells and T-cells [123]. Immature DC treated with Hsp70 upregulate expression of

co-stimulatory molecules, and show increased capacity to activate peptide-specific T-cells [205]. In vivo experiments have demonstrated Hsp70's importance in initiating an immune response to a self-antigen, switching from tolerance to autoimmune reactivity [136]. The biologic significance of anti-Hsp70 antibodies blocking Hsp70 function has been demonstrated in vivo when the antibodies prevented development of allergic contact hypersensitivity [130]. Our patient group consisted of MG patients with symptoms mostly limited to the ocular and periocular muscles, and their disease phenotype could therefore be described as mild compared to the severe generalized symptoms experienced by most MG patients. Based on the articles describing the immunological properties of Hsp70 and our current results, one can picture a scenario where inhibited Hsp70 signaling by antibody binding leads to reduced DC activation causing decreased activation of lymphocytes. This would slow down disease progression and, might explain the mild generalized symptoms among the group of MG patients that we have studied and the low frequency of anti-AChR positive patients. This theory remains speculative, as we do not have scientific data to support the specific role of Hsp70 in MG pathogenesis.

METHODOLOGICAL CONSIDERATIONS

Cell culture and choice of cell types (papers I and II)

Human myoblasts were acquired using a slightly modified protocol derived from Thompson et al. [206]. The skeletal muscle biopsy was manually minced under sterile conditions, after which it was dissociated by treatment with trypsin, type IV collagenase, and bovine serum albumin (BSA). The heterogenous cell suspension, containing both fibroblasts and muscle cells, was seeded into an uncoated cell culture flask and incubated for 1 hour. The fibroblasts adhered to the surface, whereas the myoblasts would remain in the cell suspension and were subcultured on gelatin coated surfaces for up to 10 passages. Homogeneity of the cell culture was ensured based on the morphological and behavioral properties of the cells, and fluorescent labeling of the cells using a titin-antibody.

The cells started to differentiate upon reaching a high degree of confluency and when fetal calf serum was removed from the growth media and replaced with a low concentration (2%) of horse serum. Multinucleated myotubes would appear in the culture after 3 days.

During normal muscle development, the myotubes need signals from the nerve terminals to form the punctuate expression of AChRs on the muscle cell membrane. The AChR expression in humans is concentrated in the neuromuscular synapses and can reach a density of 10 000 to 20 000 receptors per μm^2, whereas the remaining sarcolemma bears <10 per μm^2. Agrin is released from the nerve terminals, and signals through MuSK to create clusters of AChRs linked together by rapsyn [207]. It may also serve as a synapse maintenance protein [208]. We differentiated the muscle cells with media not supplemented with Agrin, and hence the AChR expression in our cells did not resemble the in vivo expression pattern. The high concentration of AChRs in the NMJ makes cross binding of antibodies and complement activation more likely to occur. Such cross binding is central in the pathogenesis of MG [9]. It is therefore possible that the patient sera tested in paper I would have been more cytotoxic to the cells, if the differentiation process had been performed with Agrin stimulation of the culture. Our aim was to identify non-AChR antibody mechanisms. However, even such mechanisms may depend on AChR antibody binding and cross linking as a necessary first step.

Several other skeletal muscle cell systems exist, both primary cells and stable cell lines. We chose to prepare our own myoblast cell cultures, as the technique is relatively well established, although often with minor modifications [206, 209-211]. In addition to primary human cell lines, stable cell lines both from humans and other species are available, according to the European Collection of Cell Cultures, ECACC). These are often easier to obtain and culture, but aspects regarding phenotypic properties and inter-species reactions are of concern. Using skeletal myoblasts of human origin, any unspecific and unwanted reactions between factors in human sera and non-human cells was avoided.

The same considerations were applied when choosing a cell line for the experiments performed in paper II. Initially the cell line HL-1 cardiomyocytes [212] was chosen. This cell line, however, was from mice and its use was discontinued as all human sera had a highly cytotoxic effect. We then decided to use the Girardi cell line, which has been used widely in heart studies including cell culture models [213-216]. It is a stable cell line of human origin, retains many of the properties of human myocardium. Potential inter-species reactions is therefore avoided.

There have been reports published on contamination of the Girardi heart cell lines, even claiming that the cells actually were HeLa cells [217]. ECCAC lists the cell type as being a HeLa derivative (cat. no. 93120822). Contamination of cell lines either by microorganisms or by a foreign cell line can happen without the handler's knowledge and can have huge implications. Even though it was first described in the 1950s it remains a big problem [218]. Genetic profiling was not performed on the cells used in paper II, and thus we cannot be completely confident that the cells used in the experiments were what they were believed to be.

The use of cell culture as a model system for studying human disease has its advantages and disadvantages. On the plus side, it is generally not very labor intensive and has a relatively low cost, compared to organ culture systems and animal models. On the down side, it differs from in vivo conditions in a variety of ways: Cell culture most often include only one or two cell types, the cells are often genetically modified making them have an infinite life span, the cells are cultured in medium without all the factors normally present in the human serum, and cultures have until the later years been confined to a two dimensional space. It

is important to be aware of these limitations in order not to over interpret the results gathered from the experiments. In an in vivo setting, any modifications to the wild type genome or any treatments or noxious stimuli applied are likely to be counter weighted by other parts of the animal (or human), yielding a milder response than what would be expected if the stimuli had been applied to only one cell type grown in cell culture.

Serum samples from patients and controls (papers I-IV)

The serum samples used in all experiments were stored as 1-2 ml aliquots at -20°C. The stability of antibodies and other serum factors has been investigated by Jellum et al. [219] using serum samples stored in the Janus Serum Bank, Norway. They noted that many serum factors, antibodies in particular, are very stable in frozen serum samples stored at -25°C. Two-dimensional protein electrophoresis was repeatedly performed on serum samples stored up to ten years, and the protein pattern remained constant, with some minor changes, over the years. These observations indicate that the primary structure of the protein remains intact, but do not shed any light on the stability of tertiary protein structure or biological activity.

Storage in an ultra freezer at -80°C or in liquid nitrogen at -196°C would probably further slow down the breakdown of serum components. This type of storage would provide “more correct” results when measuring serum components. However, this aspect can partly be compensated for by storing samples from all groups tested in a similar way.

Healthy control sera were all from Norwegian blood donors. The blood donors are assumed healthy, since the regulations in Norway do not allow people with acute or chronic cardiac disease, lung disease, urinary tract infections (including sexually transmitted diseases), diseases in the central nervous system and gastrointestinal diseases to donate blood. This includes cancer and autoimmune conditions. The samples were randomly selected from all blood donors. Information regarding sex and age were not obtainable, but the sex distribution among Norwegian blood donors reflects the public in general (52.3% males and 47.2% females), and most donors (76.8%) are aged 26-55 years [220].

CONCLUSIONS

1. Sera from MG patients have a cytotoxic effect on human myoblasts in culture and are capable of inducing morphological changes. Sera from patients undergoing MG crisis were the most potent, and the factors responsible for the changes observed had a molecular weight >100 kDa.

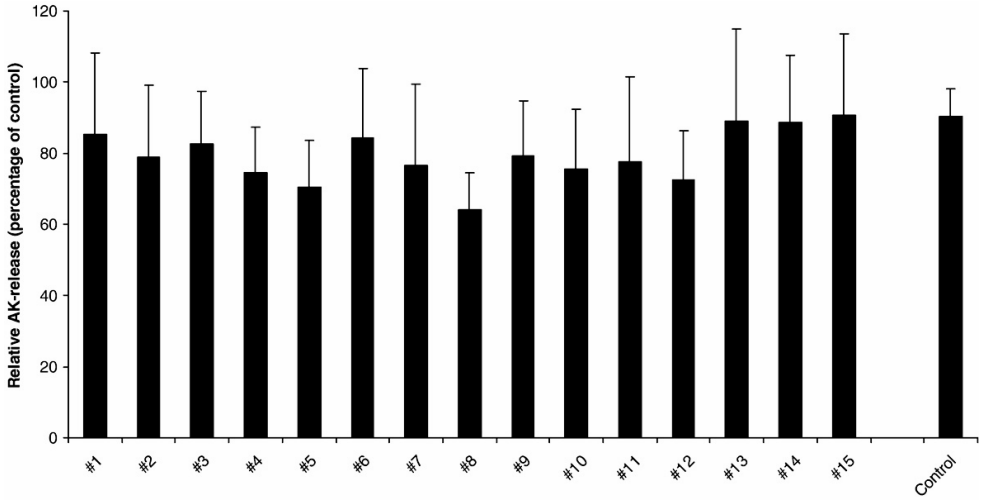
2. MG sera showed no cytotoxic potential when applied to cultured heart muscle cells.

3. MG patients with a predominance of periocular symptoms have increased levels of anti-Hsp70 antibodies, MMP2 and MMP9. No difference was found for MMP3 when comparing the entire MG group to controls, but a subgroup of MG patients had MMP3 concentrations exceeding the maximum concentration in the healthy controls.

4. Anti-Hsp70, MMP2, MMP3 and MMP9 concentrations did not differ between anti-AChR positive and negative patients or when comparing patients with purely ocular and ocular plus generalized symptoms.

ERRATA

Paper II:



The control-bar is average enzyme release from 4 healthy individuals relative to levels measured in growth medium (not stated specifically in paper II). Enzyme release from untreated cells in growth medium was set to 100%.

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