Genetic associations in myasthenia gravis

Implications for pathogenesis

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CONTENTS

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LIST OF PAPERS

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ABBREVIATIONS

Abs	Antibodies
AChE	Acetylcholine esterase
AChR	Acetylcholine receptor
Ag	Antigen
AIRE	Autoimmune regulator gene
APCs	Antigen-presenting cells
Bp	Base pair
CMAP	Compound muscle action potential
CMS	Congenital myasthenic syndromes
CT	Computed tomography
EAMG	Experimental autoimmune myasthenia gravis
E_{AP}	Treshold potential for initiating an action potential
ELISA	Enzyme-linked immunosorbent assay
E_M	Resting membrane potential
EOMG	Early onset myasthenia gravis
EPP	Endplate potential amplitude
FcγR	Fc gamma receptor
GCs	Germinal centers
HLA	Human leukocyte antigen

IL	Interleukin
INF	Interferon
LD	Linkage disequilibrium
LOMG	Late onset myasthenia gravis
MG	Myasthenia gravis
MGFA	Myasthenia Gravis Foundation of America
MHC	Major histocompatibility complex
MIR	Main immunogenic region
MuSK	Muscle specific tyrosine kinase
NMJ	Neuromuscular junction
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
RNS	Repetitive nerve stimulation
RyR	Ryanodine receptor
SF	Safety factor
SFEMG	Single fiber electromyography
SIDS	Sudden infant death syndrome
SNMG	Seronegative myasthenia gravis
SNP	Single nucleotide polymorphism

TNF	Tumour necrosis factor
T_{reg}	Regulatory T cells

INTRODUCTION

MYASTHENIA GRAVIS

EPIDEMIOLOGY

Myasthenia gravis (MG) is an autoimmune disorder, most often caused by pathogenic antibodies (Abs) against the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction (NMJ) [1]. The clinical syndrome was probably first described by Sir Samuel Wilks in 1877, in a woman initially thought to be suffering from hysteria presenting with generalized weakness, squint and dysphagia [2]. The term “myasthenia gravis” was coined by Friedrich Jolly in 1895.

Although both incidence and prevalence have increased over time, MG is still a relatively rare disease. In 1984, the prevalence of MG in Norway was reported to be 90 per million [3]. In 2007, the estimated prevalence of seropositive MG in Norway was 126.2 per million, with a yearly incidence of 7.2 per million for the period 1995 - 2008. Taking into account a 15% stipulated portion of seronegative MG (SNMG) patients, the total MG prevalence was estimated to 145 per million [4]. Globally, a prevalence between 100 and 200 per million is found in most populations studied [5], while the reported incidence varies widely between 1.7 and 10.4 per million [1].

MG affects both sexes at all ages, but in women there is a peak in incidence during early adulthood (age < 40 years). The incidence has been reported to be equal in both sexes during puberty and at older ages [4], but other investigators report a higher incidence in men above age 50 [6]. There is evidence of MG being underdiagnosed in old age (> 80 years), and this has been attributed to a wider range of differential diagnoses in this age group, with symptoms being interpreted as stroke or motor neuron disease [7].

CLINICAL FEATURES

Being a disorder of the NMJ, MG causes purely motor symptoms. Classically, patients present with fatigueable muscle weakness, involving specific muscle groups or being generalized. Most affected individuals experience fluctuation of weakness from day to day, or even from hour to hour. Weakness and fatiguability worsens with activity and improves with rest [1, 5]. Ocular symptoms in the form of ptosis and/or diplopia are the commonest initial presentation, and are seen in 85 percent of MG patients. Of these, 80 percent eventually progress to generalized weakness, most within 1 year of disease onset [6]. Maximum severity is reached within 2 years in most patients.

Prominent bulbar symptoms with dysarthria, dysphagia and facial weakness are more common in patients with antibodies against the muscle specific tyrosine kinase (MuSK) [8-9]. Respiratory muscles may be affected in MG, sometimes to a degree necessitating assisted ventilation. Such respiratory crises are more common in MuSK-positive MG. Of notion, weakness of ankle dorsiflexion is unusual in MG but not in congenital myasthenic syndromes (CMS), and so can be useful as a distinguishing feature [10].

MG SUBGROUPS

MG is usually divided into subgroups according to age at onset, thymic pathology and antibody profile. This subdivision of patients is of clinical importance, as treatment decisions are based upon it.

OCULAR MG

This subgroup is characterized by purely ocular symptoms, and comprises 17 percent of the total MG population. Some of these patients will eventually develop generalized disease, but if this does not occur within 2 years of disease onset, there is only a 10 percent risk that they will do so later on [6]. Ocular MG can affect all age groups. Abs to AChR are detected in 50 percent. Abs to MuSK are rare in ocular MG, but an association with Abs against acetylcholine esterase (AChE) have recently been found [11].

EARLY ONSET MG (EOMG)

These patients have disease onset before 50 years of age, no thymoma is present and Abs against AChR are detectable. Titin Abs are found in 10 percent [12], but Abs against the ryanodine receptor (RyR) are very rare. The EOMG subgroup has a female:male ratio of about 2.5:1 [6]. Most affected individuals have thymic hyperplasia [13].

EOMG with thymic hyperplasia is associated with HLA-DR3 and B8 [14]. These alleles are part of the conserved 8.1 HLA haplotype, which also includes HLA-A1. The 8.1 HLA haplotype is also associated with several other autoimmune diseases, such as autoimmune thyroid disease, rheumatoid arthritis and systemic lupus erythematosus [14-15]. These diseases co-occur with markedly increased frequency in MG patients [16], suggestive of shared genetic susceptibility. A protective effect of HLA-DR7 has been reported for EOMG with thymic hyperplasia [17].

LATE ONSET MG (LOMG)

Onset of disease is after 50 years of age, no thymoma is present. For LOMG there is no sex preponderance. All patients have Abs against AChR, about 60% have

additional titin Abs and 14% have also RyR Abs [12]. The course of the disease is often more severe in LOMG as compared to EOMG [18], particularly in patients with Abs against titin and RyR [19].

The thymus is usually normal or atrophic. There is an association with HLA-A3, B7, DR2 [20] and HLA-DR4 [21]. The presence of titin Abs is associated with HLA-DR7, and these patients also have a low frequency of the HLA-DR3 allele [17].

THYMOMA MG

Herman Hoppe, in 1892, speculated that a mass he found at autopsy near a large bronchus had caused auto-intoxication in a patient presenting with myasthenia [22]. About 10 percent of MG patients have a thymoma [6], while 30-50 percent of thymoma patients develop MG, which in these patients is considered a paraneoplastic disease [23]. In nearly all cases symptoms of MG precede the detection of the thymoma, which usually is otherwise asymptomatic [6, 24]. Thymoma MG occurs in all age groups, but with a peak onset around 50 years. Incidence is not influenced by gender [24], and the disease course is similar to LOMG [25-27].

Although associations with HLA-DQB1*0604 [28] and HLA-DRw15, Dw2 [21] have been reported in thymoma MG, this has not been reproducible. A protective effect of the 8.1 HLA haplotype has been suggested [29]. All thymoma MG patients have Abs against AChR. Titin and RyR Abs are found in 95 and 70 percent, respectively [12].

MuSK AB-POSITIVE MG

In 2001, Hoch et al. described for the first time MuSK as a target for auto-Abs in MG patients without detectable Abs against AChR, and also demonstrated their functional effects on agrin-induced AChR clustering [30]. MuSK Abs have been detected in

only one patient with AChR Abs [31-32]. The proportion of MuSK Ab-positive patients among the AChR Ab-negative patients varies widely from 47% in Italy, 22% in the Netherlands, to 4% in Taiwan [8, 33-34]. In Norway, having a population of 4.8 million, only three patients with MuSK Abs have been identified (unpublished data).

An association with HLA-DR14 and DQ5 has been found in MuSK Ab-positive MG [35]. Standard therapy for MG is often less satisfactory in patients with MuSK Abs, but these patients usually respond well to additional therapy with mycophenolate, cyclosporine or cyclophosphamide [31].

MG WITH LOW-AFFINITY AChR ANTIBODIES

The observation that MG patients without detectable Abs against AChR or MuSK resemble AChR Ab-positive MG both clinically and in the response to treatment [8-9, 36-37], made it likely that apparent seronegativity was due to failure of current assays to detect the Abs.

Indeed, using human embryonic kidney cells expressing clustered AChR, low-affinity Abs against AChR have been detected in 66 percent of MG patients who were negative for Abs against both AChR and MuSK using standard assays [38]. These low-affinity Abs are mainly of the IgG1 subclass and are able to activate complement, supporting their role in MG pathogenesis.

SERONEGATIVE MG

A proportion of MG patients remain without detectable Abs using both conventional and experimental assays, and these patients are referred to as seronegative. Nevertheless, there is substantial evidence that humoral factors are involved; the disease can be transferred vertically and to mice (both by plasma and immunoglobin

preparation) and patients improve after plasma exchange [39]. Both IgG and non-IgG (probably IgM) plasma factors may be important in causing the disease. Yet unidentified antigens on the postsynaptic membrane may be the target of pathogenic Abs. Also, some patients could be misdiagnosed due to the broad differential diagnosis of MG including Lambert-Eaton myasthenic syndrome, CMS, motor-neuron disease, inflammatory neuropathies and myopathies.

PATHOPHYSIOLOGY

MG is prototypical both as an Ab-mediated autoimmune disease and as a disorder of neuromuscular transmission. In MG (and all other neuromuscular transmission disorders) the safety factor (SF) for neuromuscular transmission is compromised, eventually leading to transmission failure. The SF can be defined as

$$SF = (EPP)/(E_{AP} - E_M)$$

where EPP is the endplate potential amplitude, E_{AP} is the threshold potential for initiating an action potential and E_M is the resting membrane potential [40]. As is evident, $E_{AP} - E_M$ equals the amount of depolarization needed to reach threshold. In MG, there is loss of both AChR and postsynaptic Na^+ channels, compromising the SF by reducing the EPP and increasing E_{AP} [41-42].

AChR ANTIBODIES

The main antigen in MG is AChR located at the postsynaptic side of the NMJ, and AChR Abs are detected in about 85 percent of patients with generalised disease using routine assays [43]. Such Abs were described for the first time in 1976 by Lindstrøm et al. [44].

AChRs represent cation channels composed of 5 subunits. In muscle there are two subtypes of AChR, fetal and adult, which differ in one subunit as illustrated in fig. 1.

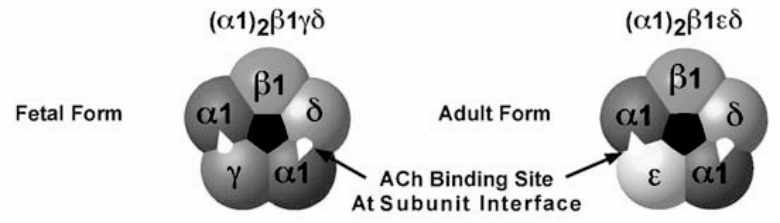


Figure 1: Composition of fetal and adult AChRs. From Kaminski (Ed.): Myasthenia gravis and related disorders [45].

Each AChR has two acetylcholine binding sites, located at the interface between α1 and the adjacent subunit [46]. The cytoplasmic domain is linked to the cytoskeleton through interaction with rapsyn, this being essential for clustering of the receptors at the motor endplate [47]. Abs against AChR bind predominantly to a region on the extracellular tip of the α1 subunit known as the main immunogenic region (MIR) [48-50], which is distant from the acetylcholine binding sites. Binding of Abs to the MIR is highly dependent on AChR being in its native conformation, suggesting that the MIR is a cluster of epitopes adjacent only in the native conformation.

Abs against AChR impair neuromuscular transmission by 3 mechanisms:

1. Antigenic modulation, which represents an increased rate of internalization and degradation of AChR due to cross-linking of the receptors by divalent Abs, thus reducing the number of AChR in the postsynaptic membrane [51-52].
2. Complement-mediated lysis of the motor endplate, resulting in simplification of the folded pattern of the postsynaptic membrane with loss of both AChR and voltage-gated Na^+ channels [42, 53-55]. As AChR loss due to antigenic modulation may be partly offset by an increased AChR synthesis [56-57], complement-mediated lysis is regarded as the most important mechanism for transmission failure in MG [58].
3. Reversible blockade of AChR by Abs directed against the acetylcholine binding sites [59-60].

A correlation between AChR Ab titer and disease severity has not been found [44], but in an individual patient fluctuations in the clinical state are often accompanied by parallel changes in the Ab titer [61].

MuSK ANTIBODIES

MuSK, by mediating agrin-induced clustering of AChR, is essential for development of the NMJ [62]. Abs against MuSK inhibit agrin-induced AChR clustering, indicating a possible effect also on maintenance of the NMJ, as MuSK is expressed at the mature NMJ [30]. Muscle weakness, electromyographical evidence of a neuromuscular transmission defect and reduced AChR clustering have been demonstrated in animal models with active MuSK immunization or passive transfer of IgG from MuSK Ab-positive MG patients [63-65].

MuSK Abs are predominantly IgG4, but they have also been shown to be of the IgG1 subclass with the ability to activate complement [38]. Although the pathogenic mechanisms are still not clear, it seems that MuSK Ab-positive MG represents an immunologically distinct entity.

OTHER ANTIBODIES

A diversity of auto-Abs have been reported in MG, including Abs against myofibrillar proteins (myosin, actomyosin, tropomyosin, α-actinin and actin) [66], the M1 muscarinic acetylcholine receptor [67], β-adrenergic receptors [68] and non-muscle antigens like interferon-α (INF-α) and interleukin-12 (IL-12) [69].

Titin is present in both skeletal and heart muscle, where it comprises the so-called third filament. It is the largest known protein to date, and produces passive force in striated muscle [70]. In 1990, Aarli et al. demonstrated that one group of non-AChR Abs found in the sera of thymoma MG patients was directed against titin [71].

RyR is a calcium release channel of the sarcoplasmic reticulum and has an essential role in excitation-contraction coupling in striated muscle [72]. The RyR1 isoform predominates in skeletal muscle, while the RyR2 isoform is the most abundant in heart muscle. Abs against RyR bind to both of these isoforms, and it has been demonstrated that they inhibit calcium release in vitro [73]. As already described, RyR Abs are mostly found in thymoma MG [12].

Although both titin and RyR Abs are able to activate complement in vitro [74], there is no evidence that these Abs are pathogenic in vivo [75]. Since both titin and RyR are located intracellularly, generation of Abs against them may be secondary to muscle damage caused by AChR Abs, although the high specificity for thymoma MG makes this less likely.

THYMIC INVOLVEMENT

Abnormalities of the thymus, and especially thymic tumours, were found to be associated with MG over hundred years ago. Improvement of MG following removal of a non-tumorous thymus was observed for the first time in 1911 [22]. Since this, several lines of evidence support an important role for the thymus in MG pathogenesis.

Muscle-like cells known as thymic myoid cells are found in the thymic medulla both normally and in MG. These cells express AChR but not MHC class II, making it unlikely that they themselves are able to present antigen (Ag) to $CD4^+$ T cells [76]. However, professional antigen-presenting cells (APCs) could present AChR from myoid cells to AChR-specific $CD4^+$ T cells. In the hyperplastic thymus of EOMG, perivascular infiltrates harbouring many APCs are frequent. Myoid cells are often located adjacent to, or within, these infiltrates [13].

Germinal centers (GCs) with AChR-specific B cells undergoing clonal proliferation, somatic hypermutation and selection are also present in the thymic infiltrates, and follicular dendritic cells in these GCs present AChR on their dendritic processes [77].

AChR Abs in MG are polyclonal in origin and belong to different IgG subclasses, implying that helper T cells are involved in the autoimmune response. Such AChR-specific $CD4^+$ T cells have been demonstrated in MG patients [78], and it has also been shown that they are more abundant in the thymus than in the blood [79-80]. However, AChR-specific T cells are also found in the blood of healthy individuals [81-82], so that in the normal situation these cells must be under tight regulatory control. Functional defects of thymic $CD4^+CD25^+$ regulatory T (T_{reg}) cells have been reported in MG [83].

Medullary thymic epithelial cells are known to play an essential role in negative selection of self-reactive T cells through expression of virtually all self-Ags of the human body, a property known as promiscuous gene expression [84]. A single nucleotide polymorphism (SNP) in the promoter region of the *CHRNA1* gene (encoding the α subunit of AChR) is associated with especially early onset of MG [85]. The MG-associated allele abrogated *CHRNA1* promoter activity in thymic epithelial cells in vitro. Furthermore, both the *CHRNA1* promoter variant and the autoimmune regulator gene (*AIRE*) was shown to modulate *CHRNA1* mRNA levels. Downregulation of *CHRNA1* expression in thymic epithelial cells could compromise negative selection of AChR-specific T cells.

A two-step model for thymic autosenitization in MG has been proposed [86]. In the first step, AChR-specific T cells are primed by APCs and thymic epithelial cells. Secondly, myoid cells are attacked, eventually provoking local GC formation in the thymus leading to the production of high-affinity AChR Abs.

In SNMG thymus, changes similar to those seen in EOMG are frequent, suggesting that these patients belong to the same etiologic group. In MuSK Ab-positive MG,

thymic changes are minimal, supportive of a different pathogenic mechanism in these patients [13].

PARANEOPLASTIC MG

Thymomas are neoplasms arising from the thymic epithelium and are classified according to the World Health Organization histological classification system. During T cell development, $CD4^-CD8^-$ T cell precursors migrate to the thymic cortex, where they develop into $CD4^+CD8^+$ T cells. These double-positive cells subsequently undergo positive and negative selection, before they differentiate into mature $CD4^+$ or $CD8^+$ T cells in the thymic medulla [87]. Only thymomas with a significant number of $CD4^+CD8^+$ T cells and also capable of exporting $CD4^+CD8^-$ T cells to the periphery are associated with MG [23], indicating that these thymomas retain the ability to propagate the maturation of T cells.

Thymoma epithelial cells express several epitopes cross-reactive with muscle proteins including AChR, titin and RyR [88-89], and their ability to present AChR peptides to T cells from patients with paraneoplastic MG has been demonstrated [90]. Both the expression of *AIRE* and the number of T_{reg} cells are low in thymomas [91], and there is a selectively reduced export of T_{reg} cells to the periphery [92]. MHC II expression is lower in thymomas than in the normal thymus. It might therefore be that T cells with high affinity T cell receptors, which normally are deleted during negative selection, instead survive [87]. In summary, it seems that mechanisms affecting both positive and negative selection are important in thymoma MG pathogenesis.

In addition to MG, many other Ab-mediated paraneoplastic disorders are associated with thymoma, such as acquired neuromyotonia, limbic encephalitis and stiff person syndrome [93]; if the aforementioned mechanisms are responsible for thymoma-associated autoimmunity, it seems reasonable that the autoimmune response is not always entirely restricted to AChR.

DIAGNOSIS

Diagnostic tools in MG include clinical examination, pharmacological tests, electrophysiological measurements, tests for detection of auto-Abs and imaging.

CLINICAL EXAMINATION

Variation in the degree and distribution of muscle weakness on repeated examinations can be helpful in making the clinical diagnosis of MG. Deep tendon reflexes are typically normal, and skin sensation is intact. Differentiating between non-specific, generalized fatigue (which is common in the general population) and the objective fatigability and weakness of specific muscles characteristic of MG is pivotal. Affected muscles are tested, and the ptosis-test should be performed. Slurred speech due to tongue weakness may only be apparent after prolonged talking. Involvement of respiratory muscles should be thoroughly sought.

Table 1 *MGFA Clinical Classification*

Class I	Any ocular muscle weakness May have weakness of eye closure All other muscle strength is normal
Class II	Mild weakness affecting other than ocular muscles May also have ocular muscle weakness of any severity
IIa	Predominantly affecting limb, axial muscles, or both May also have lesser involvement of oropharyngeal muscles
IIb	Predominantly affecting oropharyngeal, respiratory muscles, or both May also have lesser or equal involvement of limb, axial muscles, or both
Class III	Moderate weakness affecting other than ocular muscles May also have ocular muscle weakness of any severity
IIIa	Predominantly affecting limb, axial muscles, or both May also have lesser involvement of oropharyngeal muscles
IIIb	Predominantly affecting oropharyngeal, respiratory muscles, or both May also have lesser or equal involvement of limb, axial muscles, or both
Class IV	Severe weakness affecting other than ocular muscles May also have ocular muscle weakness of any severity
IVa:	Predominantly affecting limb and/or axial muscles May also have lesser involvement of oropharyngeal muscles
IVb	Predominantly affecting oropharyngeal, respiratory muscles, or both May also have lesser or equal involvement of limb, axial muscles, or both
Class V	Defined by intubation, with or without mechanical ventilation, except when employed during routine postoperative management. The use of a feeding tube without intubation places the patient in class IVb.

After the diagnosis of MG is made the patient should be classified according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification (table 1) [94]. The most severely affected muscles should be used to define the patient's current clinical class. The maximum severity experienced

during the pre-treatment period should also be recorded and used as a reference point.

PHARMACOLOGICAL TESTS

Edrophonium chloride is an acetylcholinesterase inhibitor, prolonging the action of acetylcholine at the NMJ and thereby increasing the amplitude and duration of the EPP. It has a rapid onset (30 seconds) and short duration (5-10 minutes) of action. In the edrophonium test (also known as the Tensilon test), edrophonium chloride is administered intravenously and improvement in muscle strength is observed. Resolution of ptosis or improvement in the strength of a single extraocular muscle are considered the most reliable endpoints. This test has a diagnostic sensitivity of 70 – 95 percent for generalized MG [95], but a positive response to edrophonium has also been reported in several other conditions and even in healthy controls. Although serious complications are rare, atropine should be readily available in case the patient develops severe bradycardia.

A therapeutic trial with the orally administered acetylcholinesterase inhibitor pyridostigmine (Mestinon) for some days may also be of help in the diagnostic process [5].

ELECTROPHYSIOLOGICAL MEASUREMENTS

In disorders of the NMJ, repetitive nerve stimulation (RNS) and single fiber electromyography (SFEMG) are used both to confirm the diagnosis, and also to exclude other defects of the motor unit.

RNS causes depletion of ready releasable acetylcholine in the nerve terminal, leading to failure of neuromuscular transmission in a proportion of NMJs. Thus, fewer muscle fibers contribute to the compound muscle action potential (CMAP) resulting in a progressive decrease (decrement) in amplitude. A decrement of more than 10 percent is usually considered abnormal. The characteristic finding in MG is a “U”-shaped envelope pattern due to a decremental response with partial repair after the

third or fourth response of the train [95]. The sensitivity of RNS has been reported to be 53-100% for generalized MG and 10-29% for ocular MG, with specificities of 97% and 94%, respectively [96-97]. To obtain maximal diagnostic yield several muscles should be tested, and in particular those that are clinically weak.

SFEMG is the most sensitive test for defects in neuromuscular transmission, and can demonstrate abnormalities even in clinically unaffected muscles. The neuromuscular jitter is the variation in latency from nerve activation to muscle action potential, and is quantified by measuring variation in the time interval between the action potentials of two muscle fibers belonging to the same motor unit. The sensitivity of SFEMG have been reported to be 75-99% for generalized MG and 62-99% for ocular MG, with specificities of 96-98 % and 73-96%, respectively [96-97]. Studies of nerve conduction velocity and conventional electromyography should be done to exclude other primary disorders of nerve and muscle whenever SFEMG is abnormal [95].

DETECTION OF AUTO-ABs

AChR Abs are routinely measured in a radioimmunoassay (RIA) with ^{125}Iα-bungarotoxin-labeled AChR as Ag [98]. Abs are detectable in 85 percent of generalized MG patients using this assay, and their presence verifies the diagnosis. Recently, a new assay for detection of AChR Abs has been developed [38]; this utilizes clustered AChR on the surface of transfected human embryonic kidney cells as Ag, and has the capacity to detect low-affinity Abs in a proportion of MG patients formerly negative for Abs to both AChR and MuSK.

MuSK Abs are routinely measured in a RIA with ^{125}I-MuSK as Ag, or in an enzyme-linked immunosorbent assay (ELISA) with the extracellular domain of MuSK as Ag. A cell-based assay similar as that for AChR has also recently been developed [99]. Testing for MuSK Abs should be done in all patients negative for Abs against AChR [1].

Titin Abs are detected in an ELISA with the titin fragment MGT-30 as Ag [100-101], and this assay is available for commercial use. RyR Abs are detected by western blot using crude sarcoplasmic reticulum as Ag [102]. In a patient positive for Abs to both titin and RyR the probability of a thymoma is 70 percent, and so these Abs can be used as serological markers for paraneoplastic MG [12].

IMAGING

Investigation for a thymoma by radiographic examination of the chest should be done in all patients with a confirmed diagnosis of MG. Computed tomography (CT) has an overall sensitivity of 87.1% for detecting thymic pathology, the sensitivity for thymoma and thymic hyperplasia being 88.5-97.1% and 36-71.4%, respectively [103-104]. As magnetic resonance imaging is equal or inferior to CT in the diagnosis of most anterior mediastinal tumors, including thymoma, CT should be the initial modality of choice [105].

TREATMENT

Resemblance of MG with curare poisoning was noted by both Jolly and Herman Oppenheim. In 1934, Mary Broadfoot Walker successfully relieved the symptoms of MG with the curare-antidote physostigmine, in 1935 with oral neostigmine [106].

SYMPTOMATIC DRUG TREATMENT

Orally administered acetylcholinesterase inhibitors (most often pyridostigmine) are the initial treatment in MG, and may also be used alone as long-term treatment in milder cases. These drugs are purely symptomatic. As the concentration of acetylcholine increases also at muscarinic synapses adverse effects related to this may occur, the common ones being gut hypermotility, increased sweating, excessive respiratory and gastrointestinal secretions and bradycardia [107].

IMMUNOSUPPRESSIVE DRUG TREATMENT

Immunosuppressive treatment aims at inducing and then maintaining remission. Oral corticosteroids induce remission or marked improvement in 70-80 percent of MG patients, and should be the first-line immunosuppressive treatment [107]. A temporary exacerbation of MG may occur 4-10 days after initiation of steroid treatment, and so the dose should initially be low and then gradually increased. After remission is induced, steroids should be tapered to the minimum effective dose due to the high risk of adverse effects.

Azathioprine is a purine antimetabolite which interferes with T cell function. When long-term immunosuppressive treatment is needed, azathioprine should be started together with corticosteroids to allow tapering of the latter to the minimum dose [107]. The combination of these two drugs are both more effective and better tolerated than steroids alone [108]. The therapeutic response to azathioprine can be delayed for 4-12 months, with maximal effect occurring after 6-24 months. About 10 percent of patients experience flu-like symptoms or gastrointestinal disturbances. Liver enzymes and blood cell counts should be monitored as hepatitis and cytopenias are possible adverse effects.

Other immunosuppressive drugs for the treatment of MG include mycophenolate mofetil, ciclosporin, cyclophosphamide, tacrolimus and methotrexate. These drugs should be considered in patients unresponsive or intolerant to steroids and azathioprine [107]. The use of monoclonal Abs directed against lymphocyte subsets is a promising approach for the treatment of MG. Good clinical outcome has been reported both for anti-CD20 (rituximab, a B cell inhibitor) [109-112] and anti-CD4 (a T cell inhibitor) [113], but more evidence is needed [107].

PLASMA EXCHANGE AND INTRAVENOUS IMMUNOGLOBULIN

Plasma exchange works by removing Abs from the patient's serum. Improvement is seen within the first week, lasting 1-3 months. Several case-series have shown a beneficial effect in MG [114], and plasma exchange are recommended as a short-term treatment in severe cases and in preparation for surgery [107]. Intravenous immunoglobulin are equally effective as plasma exchange in MG exacerbations [115].

THYMECTOMY

No randomized controlled trials evaluating the effect of thymectomy for non-paraneoplastic MG have been performed, but a trial is in progress [116]. In paraneoplastic MG thymectomy is always indicated irrespective of MG severity, with the aim of treating the tumour [107].

EOMG patients with generalized disease and persistent symptoms despite the use of acetylcholinesterase inhibitors are usually considered for thymectomy early on in the disease course. In LOMG, thymectomy is only recommended for the minority of patients with a hyperplastic thymus resembling EOMG. LOMG patients with titin Abs usually do not improve after thymectomy [5]. Thymectomy is not performed in ocular MG, as no beneficial effect is seen in these patients compared to medical treatment only [117-118]. In MuSK Ab-positive MG remission rates are low following thymectomy [8, 119], which therefore is not usually performed. There is no general agreement regarding the role of thymectomy for SNMG [107], but a similar postoperative course as for seropositive MG has been reported [120-121]. Negative health effects of thymectomy have never been found [5].

GENETICS OF MYASTHENIA GRAVIS

Several lines of evidence demonstrate the key role of genetic factors in MG. Up to 4 percent of patients' family members develop MG themselves [122]. There is an excess of other autoimmune diseases among family members of MG patients [14], as well as in affected individuals themselves [16]. The co-occurrence of multiple autoimmune diseases suggests shared susceptibility factors. Twin studies have shown the concordance of MG to be significantly higher in monozygotic as compared to dizygotic twins [123], strongly suggestive of a genetic predisposition.

GENETIC LOCI ASSOCIATED WITH MG

Several genetic loci, including both MHC and non-MHC genes, have been reported to be associated with MG (reviewed in [14]). Some of these will be described here.

HLA

The most reproducible genetic association in MG is the HLA-A1, B8, DR3 haplotype with EOMG with thymic hyperplasia. Several studies have shown that the predominant association is with the HLA-B8 allele [20, 124-126]. A susceptibility locus, MYAS1, has been mapped to a 1.2 Mb region comprising the distal MHC III and proximal MHC I, including *TNFA* and *TNFB* [126]. It has also been reported that HLA-DR7 confers a protective effect in EOMG with thymic hyperplasia [17], with significant peaks of negative association in the TNF gene cluster and the HLA-A locus [14].

For LOMG, associations with HLA-A3, B7, DR2 [20] and HLA-DR4 [21] have been reported. Also, titin Ab-positive patients have an association with HLA-DR7 [17]. Paraneoplastic MG is associated with HLA-A25, and for patients with a B2 thymoma HLA-A2 shows a protective effect [127]. A protective effect has also been suggested for the 8.1 HLA haplotype in paraneoplastic MG [29]. In MuSK Ab-positive patients, there is an association with HLA-DR14, DQ5 [35].

FCGR

The genes encoding Fc gamma receptors (FcγR) are clustered on the long arm of chromosome 1. Two studies have investigated whether functional polymorphisms in *FCGR2A* (encoding FcγRIIa), *FCGR3A* (encoding FcγRIIIa) and *FCGR3B* (encoding FcγRIIIb) are associated with MG; the first study reported a high frequency of the FcγRIIa 131H/H genotype in thymoma MG patients [128], while the second study found a high frequency of the FcγRIIa 131R/R genotype among MG patients, but with no difference between subgroups [129].

FcγRIIa belongs to the group of activating FcγRs [130], and is found on virtually all cells of the myeloid lineage. The variant containing a histidine at amino acid position 131 (131H) has a higher affinity for IgG2 than the variant containing an arginine (131R) [131]. Because activating and inhibitory FcγRs usually are found co-expressed on the cell surface and are co-engaged by the IgG ligand, the cellular response is determined by their activation ratio [130]. Thus, polymorphisms affecting receptor affinity may well modify immune responses.

IL10

IL10, the gene encoding interleukin-10 (IL-10), is located on the short arm of chromosome 1. The expression level of IL-10 in peripheral blood mononuclear cells (PBMCs) stimulated by Con A is related to three SNPs in the *IL10* promoter [132]; G/A at position -1082, T/C at position -819 and A/C at position -592. They constitute three haplotypes (GCC, ATA, ACC), which in combination are associated with high (GCC/GCC), medium (GCC/ATA, GCC/ACC) or low (ATA/ATA, ATA/ACC, ACC/ACC) expression of IL-10.

There are also two CA repeat microsatellites designated IL10.G and IL10.R located in the *IL10* promoter [133]. MG patients with high titres of AChR Abs have an association to IL10.G allele 134, and MG patients with normal thymic histology have an association to IL10.R allele 112 [134].

TNF

TNFA (encoding tumour necrosis factor α, TNF-α) and *TNFB* (encoding TNF-β) are located in the MHC class III region, between the complement cluster and HLA-B. Two SNPs located in the promoter region of *TNFA* may influence transcription levels; -308G/A [135] and -238G/A [136]. The high expression variant *TNFA* -308A (designated TNFA*T2) is associated with HLA-A1, B8, DR3, while the low expression *TNFA* -308G allele (designated TNFA*T1) is associated with HLA-DR4 and DR6 [137]. *TNFB* contains an NcoI diallelic restriction fragment length polymorphism in the first intron; the TNFB*1 allele correlates with increased transcription of TNF-β compared to the TNFB*2 allele [138].

Titin Ab-negative MG patients (including EOMG) have an increased frequency of the TNFA*T2 and TNFB*1 alleles, while titin Ab-positive MG patients (including paraneoplastic MG) have an increased homozygote frequency of TNFA*T1 and TNFB*2 [139].

The different genetic associations among MG subgroups, along with the observed clinical and pathophysiological heterogeneity, support that subgroups represent distinct etiological entities.

CYTOKINES IN MYASTHENIA GRAVIS

Cytokines interact with each other and with the cells of the immune system in a complex network, and their effects may be pleiotropic. In experimental autoimmune myasthenia gravis (EAMG) $CD4^+$ T cells are necessary for development of the disease [140]. SCID mice grafted with blood lymphocytes from MG patients produce Abs against human AChR and develop myasthenic symptoms only if $CD4^+$ T cells are included in the graft [141]. Therefore, investigations have focused on the role of cytokines involved in $CD4^+$ T cell function.

The Th1 subset of $CD4^+$ T cells secrete pro-inflammatory cytokines such as interferon-γ (INF-γ), IL-2 and TNF-β, and are involved in activation of APCs and cell-mediated immune responses. However, Th1 cells also promote growth and differentiation of B cells producing complement-fixing Abs both in humans and rodents [142]. Th2 cells can downregulate Th1 cells and activated APCs by secreting anti-inflammatory cytokines like IL-4, IL-5 and IL-10, and they also promote growth and differentiation of B cells producing Abs that do not fix complement [142]. Given the importance of complement-mediated damage both in MG [58] and EAMG [143], Th1 cells may have a crucial role in their pathogenesis.

IL-12 is the major growth and differentiation factor for Th1 cells [142]. Several lines of evidence have demonstrated its importance in EAMG pathogenesis, including the increased propensity seen with exogenous administration of IL-12 [144], and the prevention seen with knock-out of IL-12 signalling [145]. Results regarding the principal Th1 effector cytokine, INF-γ, have been conflicting. Investigators have reported INF-γ knock-out mice both to have similar [146] and reduced [147] susceptibility to EAMG as compared to wild-type mice, while others have found such mice to be resistant to EAMG [148].

The Th2 cytokine IL-4 appears to have a protective effect against EAMG, as IL-4 knock-outs develop more severe and persisting myasthenia than their wild-type littermates [149-150]. Conversely, other Th2 cytokines seem to facilitate EAMG; Both IL-5 and IL-6 knock-out mice develop myasthenia less frequent and with less severity [151-152]. Due to its anti-inflammatory activity, IL-10 has been regarded as a possible therapeutic option in autoimmune diseases. PBMCs from MG patients show increased in vitro spontaneous secretion of AChR Abs, but not of IL-10 [153], and decreased IL-10 mRNA expression in non-stimulated PBMCs from MG patients in vitro has been reported [154]. To the contrary, both transgenic mice expressing IL-10 under control of the IL-2 promoter and mice given subcutaneous IL-10 have an increased susceptibility to EAMG [155-156]. The diverging results regarding the role

of IL-10 in MG may reflect both the methodological variation and the complex and pleiotropic effects of this cytokine.

CONGENITAL MYASTHENIC SYNDROMES

CMS are inherited, usually autosomal recessive disorders in which failure of neuromuscular transmission is caused by specific presynaptic, synaptic or postsynaptic mechanisms. Affected individuals commonly present within the first year of life [10]. A genetic diagnosis is established in about half of CMS patients, and 80 percent of these are postsynaptic [157].

During formation of the NMJ, agrin released from the nerve terminal activates MuSK, which subsequently activates rapsyn (Receptor Associated Protein of the SYNapse), a 43 kDa membrane-associated cytoplasmic protein. Rapsyn interacts directly with AChR, inducing their clustering in the postsynaptic membrane [158]. Dok-7 (Downstream Of Kinase 7), a 55 kDa cytoplasmic protein, is an indispensible player in this process; Dok-7 interacts directly with the cytoplasmic region of MuSK, and regulates its localization, activation and responsiveness to agrin [159]. By largely unknown mechanisms, the NMJ matures during the early period of postnatal life into its adult three-dimensional structure with gutters and folds in the postsynaptic membrane [158].

It is important to emphasise that CMS, being genetic disorders, do not benefit from immunosuppressive treatment. It is therefore essential to distinguish these patients from those with autoimmune neuromuscular transmission disorders, so as to prevent the inappropriate use of immunosuppressive drugs, and eventually also thymectomy.

RAPSYN CMS

Mutations in rapsyn (encoded by the *RAPSN* gene located on the short arm of chromosome 11) are responsible for 10 percent of all CMS cases [157]. Endplate studies from these patients revealed AChR and rapsyn deficiency, and a simplified morphology with loss of postsynaptic folding [160]. Depending on the specific mutation(s), the molecular mechanisms responsible for AChR deficiency in rapsyn CMS include reduction in rapsyn self-association, stability, co-localization with AChR, and reduced stability of AChR clusters [161].

The usual presentation of rapsyn CMS is at birth with hypotonia, multiple joint contractures (arthrogryposis multiplex congenita), bulbar dysfunction and the need for mechanical ventilation. During early childhood, most patients experience recurrent episodic crises with apnea precipitated by minor infections. Later, most patients have mild symptoms, although strabismus is present in the majority. Some affected individuals, however, present during late childhood or in adulthood with symptoms resembling autoimmune MG. Patients with rapsyn CMS have a positive response to cholinesterase inhibitors [162].

Although several mutations in rapsyn have been identified, in most cases the N88K (c.264C>A) mutation is present on at least one allele [10]. This is probably due to a founder effect in people of Indo-European heritage [163]. All reported patients with late-onset rapsyn CMS have at least one copy of the N88K mutation, the vast majority being homozygous [164]. The dominance of this mutation makes screening for rapsyn CMS quick and simple.

Cholinesterase inhibitors are regarded as standard pharmacotherapy, but 3,4-diaminopyridine is an option. Some patients may benefit from a combination of the two [47].

Dok-7 CMS

Mutations in *DOK7*, the gene encoding Dok-7, are increasingly recognized as a cause of postsynaptic CMS. In a recent French cohort of CMS patients, *DOK7* mutations were found in 18 percent, superseded only by mutations in *CHRNE* (47%). In four of these patients SNMG was initially suggested [165]. NMJs are small and simplified [166-167], and denervation, reinnervation and formation of ectopic NMJs occur [165]. AChR density and function are almost normal [166], but AChE activity is reduced [165]. In addition, presynaptic changes can be observed [165].

Dok-7 CMS usually presents in early childhood, although it may also present with hypotonia at birth. In a minority of patients, the initial presentation is in early adulthood [165, 168]. A waddling gait and frequent falls are typical. Weakness predominantly affects proximal muscles of both the upper and lower extremities, giving rise to a limb-girdle phenotype. Respiratory difficulties are common, and crises necessitating invasive ventilation may occur. Ocular involvement, most often ptosis, is usually present. Fluctuations in muscle weakness dependent on exercise are experienced by most patients. The disease course is often progressive, with many patients developing spinal deformities.

In the majority of patients with Dok-7 CMS, a common four basepair duplication is detected in exon 7. This c.1124_1127dupTGCC mutation has been detected at least heterozygously in all late-onset patients identified [165, 168]. The high frequency of this mutation makes screening for Dok-7 CMS feasible.

The response to treatment with cholinesterase inhibitors and 3,4-diaminopyridine is usually poor, and these drugs may even worsen the patients clinical state. However, the edrophonium test is positive in some patients, and some also improve transiently on pyridostigmine treatment [165, 168]. Treatment with ephedrine may give substantial improvement [165, 169].

AIMS OF THE STUDY

I. To investigate whether functional polymorphisms in the IL-10 promoter associate with MG (paper I).

II. To investigate whether specific combinations of allelic variants individually associated with MG synergize in predisposing to MG (paper II).

III. To investigate whether late-onset CMS caused by rapsyn or Dok-7 mutations are frequently misdiagnosed as SNMG (paper III).

SUMMARY OF RESULTS

IL10 PROMOTER POLYMORPHISMS IN MG

Since IL-10 is important in MG pathogenesis and polymorphisms in the *IL10* promoter influence the expression level of IL-10, we analyzed the distribution of these polymorphisms in MG patients and controls to determine whether they influenced MG susceptibility. The study included 64 MG patients (26 with EOMG, 20 with LOMG, 14 with thymoma MG and 4 in which subgroup had not yet been determined) and 87 blood donors as healthy controls. All patients and controls were Norwegian Caucasians.

A 587-base pair (bp) fragment of the *IL10* promoter containing the three biallelic polymorphisms located at position -592, -819 and -1082 was amplified by polymerase chain reaction (PCR) and subsequently bidirectionally sequenced. The distribution of *IL10* genotypes is shown in table 2 and figure 2.

Table 2

IL-10 haplotype distribution.

	Total MG	EO-MG	LO-MG	Thymoma MG	Titin Ab+	Titin Ab−	Controls
ACC/ACC	**8 (12.5%)** [$P=.05$]	1 (3.8%)	**4 (20.0%)** [$P=.02$]	**3 (21.4%)** [$P=.03$]	**4 (20.0%)** [$P=.02$]	1 (4.5%)	3 (3.4%)
ACC/ATA	**4 (6.3%)** [$P=.03$]	1 (3.8%)	2 (10.0%)	1 (7.1%)	2 (10.0%)	2 (9.1%)	17 (19.5%)
ACC/GCC	14 (21.9%)	6 (23.1%)	7 (35.0%)	1 (7.1%)	4 (20.0%)	8 (36.4%)	24 (27.6%)
ATA/ATA	7 (10.9%)	**5 (19.2%)** [$P=.02$]	2 (10.0%)	0	1 (5.0%)	1 (4.5%)	3 (3.4%)
ATA/GCC	18 (28.1%)	8 (30.8%)	1 (5.0%)	5 (35.7%)	4 (20.0%)	6 (27.3%)	19 (21.8%)
GCC/GCC	13 (20.3%)	5 (19.2%)	4 (20.0%)	4 (28.6%)	5 (25.0%)	4 (18.2%)	21 (24.1%)

Values are given as number of patients and controls (percentage). Total numbers differ because of incomplete data sets. Values significant at .05 when compared to controls are in bold.

We found a significantly higher frequency of the ACC/ACC genotype in MG patients when compared to controls (12.5% vs. 3.4%, P=.05). The thymoma MG subgroup also had a significantly higher frequency of the ACC/ACC genotype (21.4%) when compared to controls (P=.03), as had the LOMG patients (20%, P=.02). EOMG patients had an increased frequency of the ATA/ATA genotype when compared to controls (19.2% vs. 3.4%, P=.02).

MG patients had a significantly lower frequency of the ACC/ATA genotype when compared to controls (6.3% vs. 19.5%, P=.03). The subgroup of LOMG patients had a lower frequency of the ATA/GCC genotype (5.0%) when compared to the remaining MG patients (P=.006).

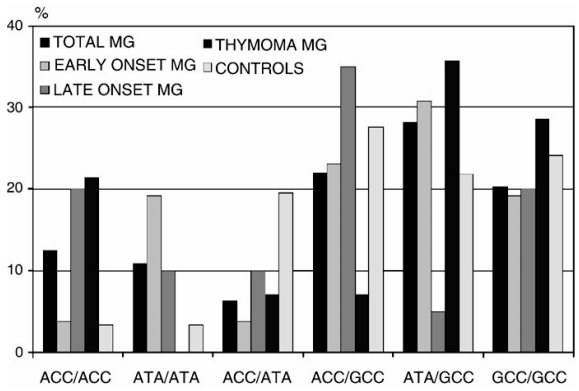


Figure 2: IL10 genotype distribution in MG subgroups and controls.

Titin Ab-status was known for 40 patients (62.5%), of which 20 were positive. Titin Ab-positive patients were similar to thymoma and LOMG patients with an increased frequency of the ACC/ACC genotype when compared to controls (20.0% vs. 3.4%, P=.02). The distribution of *IL10* genotypes in relation to titin Ab-status is shown in figure 3.

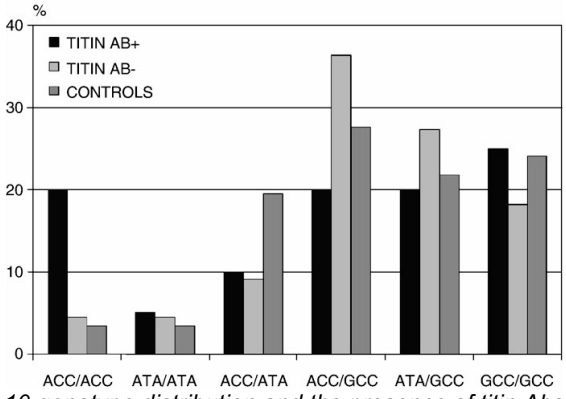


Figure 3: IL10 genotype distribution and the presence of titin Abs.

POLYGENIC DISEASE ASSOCIATIONS IN MG

Most genetic associations in MG are rather weak, and MG is probably a polygenic disease. We therefore wanted to investigate whether allelic variants in several genes, individually associated with MG, synergize in MG predisposition. The study included 47 patients with generalized MG (18 with EOMG, 19 with LOMG and 10 with thymoma MG) and 92 blood donors as healthy controls. All were Norwegian Caucasians, and none were related.

Two polymorphisms in the *TNFA* promoter were analyzed; -308G/A and -238G/A. As mentioned in the introduction, the low expression variant -308G is designated TNFA*T1 and the high expression variant -308A is designated TNFA*T2. In *TNFB* we analyzed the NcoI diallelic restriction fragment length polymorphism located in the first intron. *FCGR2A* was analyzed for the biallelic 131H/R polymorphism. Finally, we analyzed the *IL10* promoter polymorphisms located at position -592, -819 and -1082.

When comparing all MG patients with controls, MG patients had a higher frequency of the *IL10* ACC/ACC genotype (P=.01). We found no significant differences for other allelic variants, alone or in combination, when comparing all MG patients with controls.

Thymoma MG patients had a higher frequency of TNFB*2 (85.7% vs. 35.6%, P=.01) and *FCGR2A* 131H/H (55.6% vs. 22%, P=.05) when compared to controls, as shown in table 3. 55.6% (5 of 9 for which data was available) of thymoma MG patients had the 3 thymoma MG-related allelic variants TNFA*T1, TNFB*2 and *FCGR2A* 131H/H, a combination which occurred in only 6.5% of controls (P=.001) and 2.9% (1 of 34 for which data was available) of non-thymoma MG patients (P=.001). The risk of having thymoma MG correlated with the number of thymoma MG-associated allelic variants, as shown in figure 4.

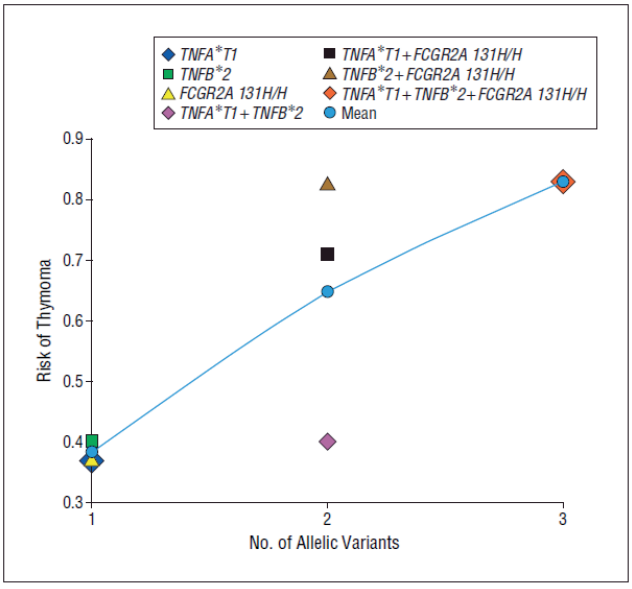
Table 3

Gene Allelic Variants in Patients With Thymomatous MG vs Control Participants and Patients With Nonthymomatous MG[a]

Allelic Variant	Patients With Thymomatous MG	Control Participants	*P* Value[b]	Patients With Nonthymomatous MG	*P* Value[b]
*TNFA*T1*	7/7 (100)	59/92 (64.1)	.09	12/23 (52.2)	**.03**
*TNFB*2*	6/7 (85.7)	32/90 (35.6)	**.01**	9/23 (39.1)	*.08*
FCGR2A 131H/H	5/9 (55.6)	11/50 (22)	**.05**	8/36 (22.2)	*.09*
IL-10 *ACC/ACC*	1/10 (10)	0/50	**1.7**	5/37 (13.5)	*>.99*
*TNFA*T1 + TNFB*2*	6/7 (85.7)	32/90 (35.6)	**.01**	9/23 (39.1)	*.08*
*TNFA*T1 + FCGR2A 131H/H*	5/9 (55.6)	9/67 (13.4)	**.009**	2/34 (5.9)	**.002**
*TNFB*2 + FCGR2A 131 H/H*	5/9 (55.6)	5/77 (6.5)	**.001**	1/34 (2.9)	**.001**
*TNFA*T1 + TNFB*2 + FCGR2A 131H/H*	5/9 (55.6)	5/77 (6.5)	**.001**	1/34 (2.9)	**.001**

Abbreviations: FCGR2A, Fcγ receptor IIa; IL-10, interleukin 10; MG, myasthenia gravis; TNFA and TNFB, tumor necrosis factors α and β, respectively.
[a]Values are given as number of patients and control participants (percentage). Total numbers of patients and control participants differ because of incomplete data sets for some.
[b]*P* values not italicized compare patients with thymomatous MG vs control participants. *P* values in italics compare patients with thymomatous MG with those with nonthymomatous MG. *P* values statistically significant at .05 are in boldface.

Figure 4



The risk of thymoma in patients with myasthenia gravis increases with the number of specific gene allelic variants considered as risk factors. The mean risk with 1, 2, or 3 specific allelic variants is also shown.

Titin Ab-positive MG patients had a similar genetic profile as thymoma MG patients, with the combination of TNFA*T1, TNFB*2 and *FCGR2A* 131H/H being found in 31.6%, compared to 6.5% of controls (P=.007) and none of the titin Ab-negative MG patients (P=.02), as shown in table 4.

Table 4

Gene Allelic Variants in Patients With Titin Ab–Positive MG vs Control Participants and Patients With Titin Ab–Negative MG[a]

Allelic Variant	Patients With Titin Ab–Positive MG	Cotrol Participants	*P* Value[b]	Patients With Titin Ab–Negative MG	*P* Value[b]
*TNFA*T1*	12/14 (85.7)	**59/92 (64.1)**	**.14**	**6/14 (42.9)**	*.046*
*TNFB*2*	10/14 (71.4)	**32/90 (35.6)**	.02	4/14 (28.6)	*.06*
FCGR2A 131H/H	7/19 (36.8)	**11/50 (22)**	**.23**	5/19 (26.3)	*.73*
IL-10 *ACC/ACC*	3/19 (15.8)	0/50	**.02**	1/20 (5)	*.34*
*TNFA*T1* + *TNFB*2*	10/14 (71.4)	32/90 (35.6)	**.02**	4/14 (28.6)	*.06*
*TNFA*T1* + *FCGR2A 131H/H*	6/19 (31.6)	9/67 (13.4)	.09	1/17 (5.9)	*.09*
*TNFB*2* + *FCGR2A 131H/H*	6/19 (31.6)	5/77 (6.5)	**.007**	0/17	***.02***
*TNFA*T1* + IL-10 *ACC/ACC*	2/18 (11)	0/67	**.04**	0/19	*.23*
*TNFB*2* + IL-10 *ACC/ACC*	2/18 (11)	0/77	**.03**	0/19	.23
*TNFA*T1* + *TNFB*2* + *FCGR2A 131H/H*	6/19 (31.6)	5/77 (6.5)	**.007**	0/17	***.02***
*TNFA*T1* + *TNFB*2* + IL-10 *ACC/ACC*	2/18 (11)	0/77	**.03**	0/19	*.23*

Abbreviations: Ab, antibodies; FCGR2A, Fcγ receptor IIa; IL-10, interleukin 10; MG, myasthenia gravis; TNFA and TNFB, tumor necrosis factors α and β, respectively.

[a] Values are given as number of patients and control participants (percentage). Total numbers of patients and control participants differ because of incomplete data sets for some.

[b] *P* values not italicized compare patients with titin Ab–positive MG vs control participants. *P* values in italics compare patients with titin Ab–positive MG with those with titin Ab–negative MG. *P* values significant at .05 are in boldface.

EOMG patients had an increased frequency of TNFB*1 (40% vs. 7.8%, P=.01) and the *IL10* ATA/ATA genotype (16.7% vs. 2%, P=.05). No combination of EOMG-associated allelic variants showed a significant difference in distribution between EOMG patients and controls, and the occurrence of more than one EOMG-associated allelic variant was rare both in EOMG patients and in controls.

RAPSYN AND Dok-7 CMS IN SNMG PATIENTS

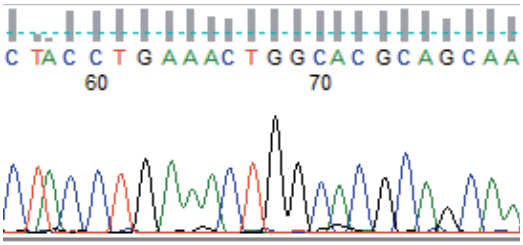
Since late-onset CMS caused by rapsyn or Dok-7 mutations can resemble autoimmune MG both clinically and electrophysiologically, we wanted to investigate the frequency of these disorders in the population of apparent SNMG patients. DNA from 76 patients diagnosed with SNMG at a neurology department in Norway was obtained from the biobank at the Department of Neurology, Oslo University Hospital, Kirkeveien. 37 blood donors participated as healthy controls.

Exon 2 of *RAPSN* and exon 7 of *DOK7* were amplified by PCR and subsequently bidirectionally sequenced. In patients heterozygous for the rapsyn N88K mutation, the promoter and remaining exons were sequenced looking for an additional mutation.

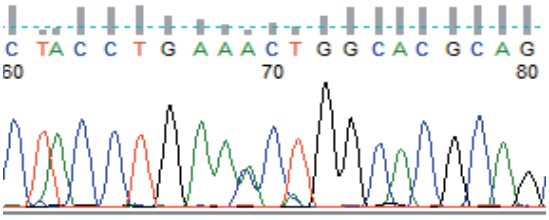
Among the 76 SNMG patients, we found one who was homozygous for the rapsyn N88K mutation, i.e. she had late-onset postsynaptic CMS (figure 5a). We also found two carriers of this mutation, one patient and one control (figure 5b).

Figure 5: Sequences showing the rapsyn N88K (c.264C>A) mutation in the a) homozygous and b) heterozygous states.

a) Homozygous N88K (A at base 65)



b) Heterozygous N88K (C+A at base 69)



Sequencing of the *RAPSN* promoter and the 7 remaining exons in the patient heterozygous for N88K revealed no additional mutation.

We reviewed the clinical data of the patient found to have late-onset rapsyn CMS. Myasthenic symptoms presented for the first time in the post partum period at age 35 years. She experienced muscle leg weakness and impaired swallowing, and later respiratory muscle weakness necessitating invasive ventilation. MG was suspected, and edrophonium and pyridostigmine had an alleviating effect. She was later thymectomized with no beneficial effect, and thymus histology was normal.

Sequencing exon 7 of *DOK7* revealed no mutations in SNMG patients or in the controls.

GENERAL DISCUSSION

Investigating MG patients for *IL10* promoter polymorphisms, we found an increased frequency of the ACC/ACC genotype in titin Ab-positive, LOMG and thymoma MG patients. This association could be related to the presence of titin Abs, given the high frequency of such Abs in LOMG and thymoma MG. The increased frequency of the ACC/ACC genotype in the total MG population when compared to controls results from the high frequency of this genotype in LOMG and thymoma MG. In EOMG patients, a higher frequency of the ATA/ATA genotype was found.

Interestingly, both the ACC/ACC and ATA/ATA genotypes are associated with low expression of IL-10 [132]. As differences in LPS-induced IL-10 secretion are related to corresponding differences in mRNA production, rather than increased mRNA stability, transcription is implicated as the principal mechanism for variation in IL-10 production [170]. Thus, the finding of low-producer *IL10* genotypes in MG points to IL-10 as a factor of importance in MG pathogenesis.

Our finding of low producer *IL10* genotypes in MG is in line with previous observations regarding the role of Th1 cells in MG pathogenesis; IL-10 is a powerful inhibitor of proliferation and cytokine production in Th1 cells both via its down-regulating effect on APCs and by a direct inhibitory effect on the Th1 cells and their IL-2 and TNF production [171]. As Th1 cells induce synthesis of complement-fixing Abs [142] and complement-mediated lysis of the muscle endplate is regarded as the most important pathogenic mechanism in MG [58], low levels of IL-10 could exaggerate the autoimmune response in MG.

IL-10 has also direct effects on B cells, and these effects are dependent upon their activation state. In B cells stimulated in vitro, IL-10 exerts suppressive influence during the initial activation, whereas it promotes an active response following activation [172]. IL-10 also inhibits IL-2 production, and IL-2 is a crucial differentiation factor for B cells [173-174].

That MG is associated with low-producer *IL10* genotypes may also explain part of the response to treatment with glucocorticoids. The *IL10* promoter contains a glucocorticoid response element motif [133, 175], and glucocorticoids up-regulate constitutive IL-10 production in human monocytes, measured at both protein and mRNA levels. This effect is abolished by the glucocorticoid receptor antagonist RU486 [176]. Glucocorticoids also increase IL-10 expression in PBMCs from multiple sclerosis patients with acute relapse [177]. An association between -1082 A/A (low producer) *IL10* genotype and steroid-dependency has been shown for ulcerative colitis and Crohns disease [178].

In paper II, the increased frequency of the *IL10* ACC/ACC genotype in the total group of MG patients was confirmed. However, the study population in paper II was also included in paper I, and so this was not a replication in an independent population. The association with the ACC/ACC genotype was also confirmed for titin Ab-positive MG patients, as was the association with the ATA/ATA genotype for EOMG. We did not find an association of the ACC/ACC genotype with LOMG and thymoma MG. However, the number of patients was lower in paper II, and so this study might have been underpowered to detect these differences in genotype distribution. Given a frequency of 3.4% for the ACC/ACC genotype in controls and an odds ratio of 7.64, a sample size of 70 in each group would be needed to give a 90% power of achieving 5% significance. Paper II also confirmed previously reported associations at the *TNFA*, *TNFB* and *FCGR2A* loci with MG subgroups [128, 139, 179].

We found that the risk of having a thymoma in patients with MG correlated with the number of allelic variants individually associated with thymoma MG. This demonstrates that thymoma MG is a polygenic disorder. It remains to be determined whether the association primarily exists with the development of MG in the population of thymoma patients or with the development of the thymoma per se.

The thymoma MG-associated allelic variants can be used as markers for the presence of a thymoma in the MG population, in which thymomas are much more common than in non-MG controls. However, a CT scan of the chest is thought to have a sensitivity of about 90-95 percent for detecting a thymoma [103-104]. In our study, all thymoma MG patients for whom records were available had preoperative findings on CT of the mediastinum indicative of a thymoma.

The genetic profile of thymoma MG patients corresponds to a phenotype with low expression of TNF-α, TNF-β and IL-10, and with optimal interaction between FcγRIIa and IgG2 [131-132, 135, 138]. Abs to AChR are predominantly of the IgG1 and IgG3 subclasses, but anti-AChR IgG2 has also been detected in MG sera [180]. IgG2 has been demonstrated to be an effective inducer of EAMG [181]. Although IgG subclasses do not directly correspond in rodents and humans, it may be that IgG2 Abs are involved in the induction of MG.

Expression of several muscle epitopes has been identified in thymomas [88-89, 182-184] and there is strong evidence for an intra-thymoma immunization process against them in paraneoplastic MG [80, 90-91]. Given that this immunization process involves IgG2, IgG2-Ag complexes will bind efficiently to FcγRIIa on APCs. Presentation of Ag epitopes to Th cells will lead to a predominantly humoral immune response, due to low levels of TNF-α and TNF-β. Furthermore, low levels of IL-10 will promote the Ag-presentation of APCs and the production of Th1-dependent complement-fixing Abs (figure 6).

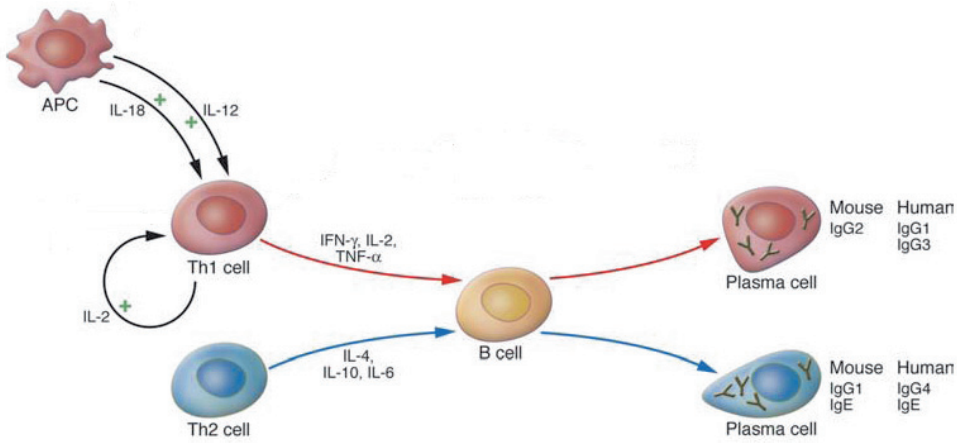


Figure 6: Production of complement-fixing Abs is dependent on Th1 cells, while Th2 cells induce the production of Abs that do not fix complement. From Conti-Fine, et al. (2008). "CD4+ T cells and cytokines in the pathogenesis of acquired myasthenia gravis." Ann N Y Acad Sci 1132: 193-209 [185].

Our data show that Titin Ab-positive MG patients have a genetic profile similar to thymoma MG. Nearly all patients with paraneoplastic MG are positive for titin Abs [12], and one might speculate as to whether non-thymoma MG patients positive for titin Abs have already rejected an occult thymoma. Titin Abs are found in about 60 percent of LOMG, but in only 10 percent of EOMG patients. Paraneoplastic MG has a peak onset around 50 years of age [23].

In EOMG, more than one EOMG-associated allelic variant rarely occurred in an individual patient. This correlates with previous findings in these patients, where the main association have been with the ancestral 8.1 HLA haplotype, which includes the TNFA*T2 and TNFB*1 alleles. A susceptibility locus termed MYAS1 has been mapped between the distal MHC III and proximal MHC I [126]. This 1.2 Mb region notably includes the TNF gene cluster. Thus, both previous observations and our study suggest that EOMG is more closely linked to a specific gene at the MYAS1 locus, rather than to a specific combination of the allelic variants tested by us. The TNFA*T2 and TNFB*1 alleles correlate with a high TNF production [135, 138], which may contribute to thymic GC formation [186].

In paper III, we screened 76 SNMG patients for the common mutations N88K (c.264C>A) and c.1124_1127dupTGCC in *RAPSN* and *DOK7*, respectively. We found one patient homozygous for the rapsyn N88K mutation. The patient underwent a thymectomy which we now know could have been avoided. This underlines the importance of considering late-onset rapsyn CMS in patients diagnosed with SNMG. The patient had strabismus and experienced deterioration of muscle weakness during intercurrent febrile illness, both typical of rapsyn CMS [10, 187-188]. Increased awareness of rapsyn CMS in the differential diagnosis of SNMG is important in order to make the correct diagnosis.

In addition, two carriers of the rapsyn N88K mutation were identified, one patient and one control. We calculated a carrier frequency of 1.8% (2 out of 112) for this mutation, which is similar to the frequency of 1.7% previously reported in 300 controls [189]. The homozygote frequency (q^2) for rapsyn N88K was estimated:

$$q = \frac{2}{224} \Rightarrow q^2 = \frac{1}{12500}$$

This estimated homozygote frequency suggests that the number of N88K homozygotes in the Norwegian population (4.8 million) is about 380. However, such a high carrier frequency of the N88K mutation has not been found in other studies [160, 162, 190]. The estimated number of N88K homozygotes may therefore be too high. In the region served by Haukeland University Hospital, having a population of 1 million, there are only 5 patients with a diagnosis of CMS (ICD: G70.2). This strongly suggests that CMS is underdiagnosed. This is also suggested by others [169], although there is no reliable epidemiological information to confirm this.

It is tempting to speculate as to whether a proportion of sudden infant death syndrome (SIDS) cases represent undiagnosed CMS. Parents of SIDS victims frequently report that their baby had symptoms of minor infection during the immediate days before death, especially respiratory tract symptoms, but these infections are not believed to

be the cause of death [191]. Most patients with rapsyn CMS experience recurrent episodic crises with apnea precipitated by minor infections [162]. One study reported presynaptic CMS due to mutations in *CHAT* (encoding choline acetyltransferase) as the underlying cause of SIDS [192]. To our knowledge there have not been any investigations for postsynaptic CMS in cases of SIDS. It would be interesting to pursue this in the future.

Screening for rapsyn CMS is greatly facilitated by the dominance of the N88K mutation. Although only one of 76 SNMG patients in our study had rapsyn CMS, we propose testing all SNMG patients for the N88K mutation before initiating immunosuppressive treatment, and before eventual thymectomy. This is justified by the significant adverse effects of unnecessary immunosuppressive treatment and the ease of screening for the N88K mutation.

We did not find any SNMG patients with the *DOK7* c.1124_1127dupTGCC mutation. *DOK7* mutations were found in 18 percent of CMS patients in a recent French cohort [165]. Four of these patients had an initial diagnosis of SNMG; three had an apparent response to steroids, two to intravenous immunoglobulin, and two were thymectomized. Thus, one should be aware of the possibility of Dok-7 CMS in patients diagnosed with SNMG.

The relatively recent discovery of *DOK7* mutations makes it likely that these will be increasingly recognized as the molecular basis for limb-girdle myasthenia of unknown aetiology in the future. Due to the high frequency of the c.1124_1127dupTGCC mutation, screening for Dok-7 CMS is feasible.

METHODOLOGICAL CONSIDERATIONS

INVESTIGATION OF GENETIC ASSOCIATIONS (PAPERS I AND II)

Genetic association studies for rare disorders like MG are case-control studies based on the comparison of allele frequencies between affected individuals and unaffected controls. A statistically significant difference indicates association of the genetic variant with the disease. An association can arise for three principal reasons:

1. The associated allele is causative for the disease.
2. The associated allele is in linkage disequilibrium (LD) with the actual causative allele.
3. A false-positive association.

False-positive associations can occur due to a number of reasons; studies without correction of p-values for multiple hypothesis testing represent a widespread problem. Determining the appropriate level of statistical significance is of particular concern in genome-wide association studies, in which hundreds of thousands of SNPs are genotyped [193]. A false-positive association can also arise because of population stratification due to ethnic admixture. If the study population is mixed and the trait investigated is present at a higher frequency in one ethnic group, then any allele that also happens to be more frequent in that group will show a positive association with the trait [194-195]. Finally, different rates of genotyping error or success between cases and controls may lead to falsely different allele frequencies.

All patients and controls included in papers I and II were Norwegian Caucasians. It is therefore unlikely that the observed associations are due to population stratification.

In paper II, p-values were not corrected for multiple hypothesis testing. It can be argued that such correction, when analyzing small samples, increases the risk of making type II errors. An increased sample size and appropriate correction of p-

values would be desirable. A multi-centre study would enable a larger sample size, but this would likely also increase genetic heterogeneity. Positive findings should always be confirmed in independent patient materials, this being especially important when small samples are examined and without correction for multiple comparisons.

The *TNFA* and *TNFB* loci, located in the MHC class III region, are in LD with each other and with other loci in the MHC region on the short arm of chromosome 6. The *FCGR2A* and *IL10* loci are located on the long and short arm of chromosome 1, respectively, and so they are not in LD with the other loci investigated in paper II. Therefore, the high frequency of multiple thymoma MG-associated alleles in individual thymoma MG patients is not merely due to LD between the investigated loci.

A major problem in association studies has been a lack of reproducibility [196]. This can be attributed to three main causes:

1. A false-positive association is correctly not replicated. This is probably responsible for a majority of non-reproducible associations [197].
2. A true association in one population is not true in a second population due to heterogeneity in their genetic or environmental background. If the investigated allele is not causal, but in LD with the causal allele, the extent of historical recombination may differ between populations. This will be reflected in the strength of association differing between the populations.
3. A false-negative follow-up study.

False-negative studies are most commonly due to an underpowered sample [195], and they are probably an important cause of the inconsistency seen in genetic association studies [197]. Due to the “winner’s curse” (a phenomenon explaining why the initial report describing an association nearly always overestimates the effect size [197]), follow-up studies should have a sample size large enough to detect a more modest effect size than initially reported. Similar to false-positive associations, false-negative results may also occur due to population admixture and technical errors.

If the sample size required in order to reach statistical significance is very large, i.e. the investigated association is weak, then the association will be of limited clinical relevance. However, such an association may still be important by pointing to the pathogenic mechanism involved.

DETECTION OF GENETIC MUTATIONS (PAPER III)

Bidirectional DNA sequencing is a sensitive method for the detection of point mutations, as well as small deletions and duplications. Deletions of whole exons, however, may easily be missed by this technique. Mutations in non-sequenced regions, e.g. intronic mutations affecting RNA splicing, will of course also be missed. To identify whole-exon deletions, quantitative PCR can be used.

In paper III, we identified one SNMG patient who was heterozygous for the rapsyn N88K mutation. Bidirectional sequencing of the *RAPSN* promoter and remaining exons, including their flanking intronic sequences, did not reveal any additional mutations. We examined the sequences for all known SNPs, as heterozygosity for these would strongly indicate the presence of two disease-inducing alleles. However, these were all present at homozygosity. We cannot exclude the possibility of a whole-exon deletion in this patient.

CONCLUSIONS

The present study provides knowledge of genetic associations in MG. The study demonstrates how functional polymorphisms in the *IL10* promoter are associated with subgroups of MG patients. The polymorphisms which associate with MG constitute low-producer *IL10* haplotypes. This may be important in MG pathogenesis. Further studies investigating the role of IL-10 are needed to elucidate the complex role of this cytokine in MG.

The risk of having a thymoma in patients with MG correlates with the number of allelic variants individually associated with thymoma MG. This demonstrates how thymoma MG is a polygenic disorder. The association could be with the development of MG among thymoma patients, or with the development of thymoma per se. Thymoma MG-associated allelic variants could be used as markers for the presence of a thymoma in the MG population. CT scan of the chest has, however, a higher sensitivity and specificity.

In EOMG, the occurrence of more than one EOMG-associated allele in an individual patient is very rare. This suggests that EOMG is more closely linked to a specific gene at the MYAS1 locus, than to a specific combination of the allelic variants tested.

Late-onset rapsyn CMS can be mistaken for SNMG even after a full examination by neurologists. However, the frequency of rapsyn CMS in our nationwide cohort of (apparent) SNMG patients is low. The carrier frequency of the rapsyn N88K mutation suggests that rapsyn CMS is underdiagnosed. There are no patients with late-onset Dok-7 CMS in our nationwide SNMG cohort. The *DOK7* c.1124_1127dupTGCC mutation is not detected in any patients or controls.

ERRATA

On pages 27 and 47 in the dissertation, *IL10* is incorrectly described as being located on the short arm of chromosome 1. The correct location of *IL10* is on the long arm of chromosome 1 (1q31-q32).

Paper I

Throughout the article, genotypes are referred to as haplotypes (e.g. ACC/ACC haplotype, which should be ACC/ACC genotype). Also, the *IL10* gene is referred to as the IL-10 gene.

Paper II

Throughout the article, the *IL10* gene is referred to as the IL-10 gene.

In table 1, the P-value for the comparison of *IL10* ACC/ACC genotype between thymoma MG patients and controls should be 0.17, not 1.7. All P-values comparing thymoma and non-thymoma MG patients should be in italics (column 6 of the table).

In table 2, several P-values >0.05 are in boldface, while some P-values <0.05 are not; only P-values ≤0.05 should be in boldface. All P-values comparing titin Ab-positive and -negative MG patients should be in italics (column 6 of the table).

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